LIVER FERRITIN IN THE PROTEIN DEFICIENT RAT

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ABSTRACT OF THESIS

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Title of Thesis Liver Ferritin in the Protein Deficient Rat

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CONTENTS

CHAPTER 1

INTRODUCTION

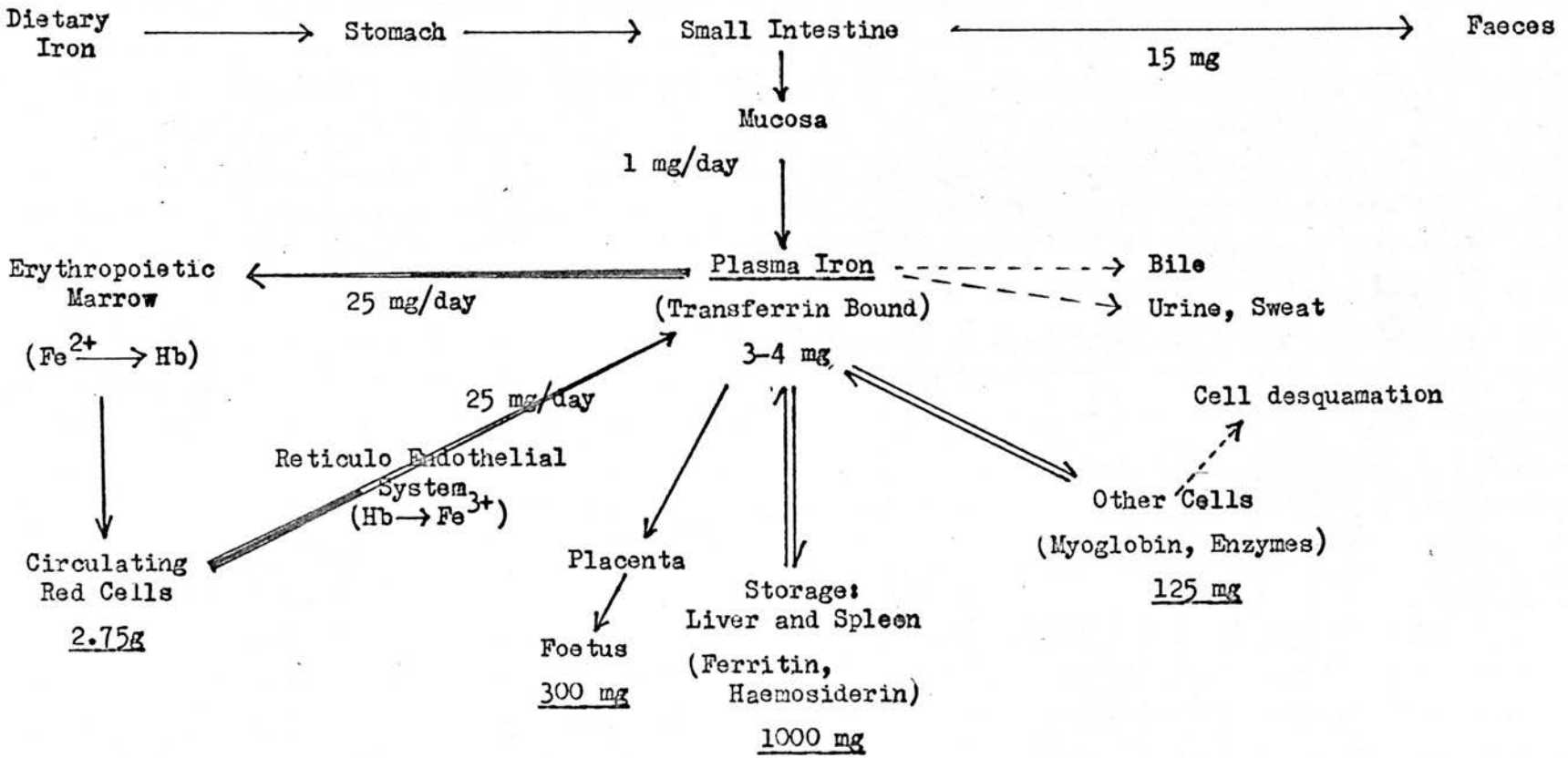
Iron Metabolism

Iron occupies a prominent role in many of the physiological processes essential for life. It is present in those iron porphyrin (haem) compounds necessary for oxygen transport and for biological oxidations (haemoglobin, myoglobin, cytochromes, catalase) and in non haem flavoproteins such as succinic dehydrogenase and xanthine oxidase. It is also present attached to the plasma transport glycoprotein, transferrin, and in storage form as ferritin and haemosiderin. A generalised scheme of iron metabolism is presented in Fig. 1.1.

The quantities illustrated represent those for the normal adult human (Van Campen 1974) but broadly similar proportions pertain to other mammalian species. Losses of iron from the body and absorption from dietary sources are both restricted (1-3 mg per day for a normal adult human). Within the body the major portion of iron is involved in haemoglobin synthesis and catabolism (25 mg per day). Destroyed red cells are processed by the reticuloendothelial cells and the iron, transported in the plasma bound to transferrin, is delivered to the erythroid marrow for incorporation into new red cells. Under normal conditions there is little exchange of iron between other tissues and the plasma (Moore 1959-60).

FIG. 1.1

Main Features of Iron Metabolism in Humans



Storage Iron

Normally 20-30 per cent of the iron of the mammalian body is stored (Drabkin 1951) as a reserve which can be drawn upon when required. For example, after acute blood loss the needs of the erythroid marrow for iron are met primarily by rapid mobilisation from the stores (Haskins et al. 1952). The iron is stored intracellularly as soluble ferritin and insoluble haemosiderin (Neumann 1888, Laufberger 1937, Hahn et al. 1943, Granick 1951, Shoden et al. 1953), the principal locations being the liver, spleen and the bone marrow. In the liver of several species including the rat there is normally a preponderance of ferritin over haemosiderin and there is evidence to suggest that iron is preferentially mobilised from ferritin (Hampton 1954, Millar et al. 1970, Wyllie and Kaufman 1971).

1. Haemosiderin

The term haemosiderin was introduced by Neumann (1888) to describe microscopically visible, insoluble iron rich granules of horse spleen. Varying opinions on the structure of the substance have been expressed (Cook 1929, Behrens and Asher 1933, Ludewig 1957, 1959, McKay and Fineberg 1958, Shoden and Sturgeon 1960, Wöhler 1960); this may well be largely a result of difficulties associated with purification. Most analysts have found that haemosiderin contains on the average more iron and less nitrogen than ferritin (Harrison 1964). Shoden and

Sturgeon (1960) suggested that the term "haemosiderin" be reserved for that water insoluble portion of tissue iron which consisted of an amorphous condensation of ferric hydroxide that was essentially protein free, but this view may be rather extreme. Recently, Vidnes and Helgeland (1973) have described a procedure in which 3000 fold purification of rat liver haemosiderin was achieved; the product of this isolation contained only 9 per cent iron, but this finding is difficult to reconcile with the observations of previous workers.

2. Ferritin

Ferritin is widely distributed throughout nature and in the human it has been identified in 12 organs (Arora et al. 1970). It was first isolated and crystallised from horse spleen (Laufberger 1937) and the protein from this source has been thoroughly investigated.

Ferritin is comprised of a protein shell, apoferritin, which encompasses variable amounts of ferric iron in the form of a hydrated ferric oxyhydroxide phosphate micelle. The iron content of the protein may vary from zero (apoferritin) up to a maximum of 4300 iron atoms per molecule of protein (Fischbach and Anderegg 1965). As a consequence of the variable iron content contained within the protein shell, electrophoretically homogeneous preparations of ferritin contain a heterogeneous population of molecules. This was first demonstrated in the analytical ultracentrifuge by Rothen (1944) who separated apoferritin

from the more dense iron containing molecules. Apoferritin can be obtained from ferritin preparations by density gradient centrifugation (Fischbach and Anderegg 1965) or by chemical removal of iron from ferritin (Granick and Michaelis 1943). There is strong evidence that the molecule of apoferritin consists of 24 subunits (Hoare et al. 1975), each of molecular weight 18500 (Bryce and Crichton 1971, Bjork and Fish 1971). The exact arrangement of the 24 subunits is not known with certainty (Crichton 1973) but a distribution with each subunit at the vertices of a snub cube (Harrison 1959) has been suggested. The apoferritin molecule can be reversibly dissociated into subunits with 67 per cent acetic acid (Harrison and Gregory 1968) and a similar dissociation has been shown to occur in the pH range 2.8-1.6 (Crichton 1972). Treatment with sodium dodecyl sulphate also brings about a reversible dissociation into subunits (Smith-Johannsen and Drysdale 1969). Some recent work suggests that the subunits are composed of multiple components (Niitsu et al. 1973, Ishitani et al. 1975). Ishitani et al. (1975) reported that human, horse, rat and rabbit ferritins invariably showed components of molecular weight 19000, 11,000 and 8000 and suggested that the 19000 molecular weight component was a composite of the 11,000 and 8000 molecular weight chains. Linder et al. (1974) have suggested that there are two types of subunits, of molecular weight 19500 and 13000,

which are synthesised independently on different polysome populations. Such findings introduce uncertainty into ideas about the quaternary structure of the complete ferritin molecule.

The diameter of the protein shell has been estimated by X-ray diffraction to be 120Å while that of the iron micelle is 70-75Å (Fischbach and Anderegg 1965). The exact chemical nature of the micelle is not known but the composition approximates $(FeO.OH)_8$. $(FeO.PO_3H_2)$ (Michaelis et al.1943, Granick and Hahn 1944). X-ray diffraction and electron diffraction studies (Harrison et al.1967) have indicated that phosphate is not an essential structural component of the micelle; its function remains unsolved. From their investigations Van Kreel et al.(1972) concluded that ferritin phosphate was in dynamic equilibrium with the phosphate of liver and spleen. Optical rotatory dispersion and circular dichroism studies have shown that both apoferritin and ferritin have substantial helical content (Listowsky et al.1972). Aggregate forms of ferritin and apoferritin have been observed on ion exchange chromatography, ultracentrifugation and gel electrophoresis (Suran and Tarver 1965, Richter 1963, Harrison and Gregory 1965). These have been attributed to the association of protein monomers to form dimers, trimers, tetramers. The oligomers have been shown to be stable against dissociation on standing for long periods or in

dilute solutions (Williams and Harrison 1968, Niitsu and Listowsky 1973, Bjork 1973) although the precise nature of the intermolecular bonding appears uncertain. Harrison and Gregory (1965) and Williams and Harrison (1968) have suggested that a disulphide linkage is not involved; Niitsu and Listowsky (1973) have presented evidence to the contrary.

Differences in electrophoretic behaviour have demonstrated the existence of species specific ferritins (Theron et al.1963, Kopp et al.1964, Richter 1965). Organ specific ferritins within single species have also been shown to exist (Allfrey et al.1967, Gabudza and Gardner 1967, Gabudza and Pearson 1969, Linder-Horowitz et al. 1970, Arora et al.1970, Linder et al.1973, Crichton et al. 1973). Ferritins from various rat or human neoplasms and the corresponding ferritin from normal tissues differ electrophoretically (Richter 1965, Richter and Lee 1970, Linder et al.1970). Evidence from isoelectric focusing has suggested a multi-component nature for tissue specific horse and rat ferritins (Drysdale 1970, Urushizaki et al.1971, Urushizaki et al.1973). This microheterogeneity has been further observed in tumor cells in which the number of components are shown to differ from that of the normal (Alpert et al.1973, Makino and Kono 1969, Marcus and Zinberg 1974). Since the metabolic significance of isoferritins is unresolved, the present work has sought to avoid involvement in this

area and the method used for isolating ferritin has been directed towards obtaining the maximum yield of this protein with the minimum possibility of fractionation.

Ferritin and apoferritin have similar electrophoretic mobilities and are equally well precipitated with a ferritin antibody (Mazur, Shorr 1950). Both proteins are comparatively stable at high temperatures and in the presence of denaturing solvents, they are also stable over a wide pH range (2.8-10.6) and heating at 80 C does not precipitate the protein from solution. Denaturation in urea (10.0 mol/ℓ) and guanidinium chloride (7.0 mol/ℓ) has been shown to depend on the pH (Listowsky et al. 1972). It has been suggested that the highly ordered secondary structure may account for the stability at elevated temperatures and in the presence of denaturing agents (Listowsky et al. 1972). Proteolytic digestion at pH 8.5 with trypsin, chymotrypsin and subtilisin has been shown to degrade apoferritin to a greater extent than ferritin; but at pH 3.0, using pepsin and cathepsin, no differences in the rate and extent of digestion were observed (Crichton 1971).

Biosynthesis of ferritin:

The biosynthesis of ferritin has been routinely measured by following the incorporation of ^{14}C-leucine into the product. Stimulation of apoferritin synthesis by administration of iron compounds has been demonstrated 'in vivo' with guinea pig and rat liver (Fineberg and

Greenberg 1955, Loftfield and Harris 1956, Drysdale and Munro 1966, Coleman and Matrone 1969, Millar et al. 1970); in rat small intestine (Bernier et al. 1970); rat intestinal mucosa (Smith et al. 1968, Cumming et al. 1970); rat heart and kidney (Linder-Horowitz et al. 1970). 'In vitro', the effect has been shown using rat liver slices (Yu and Fineberg 1965); HeLa cells (Chu and Fineberg 1969); slices of rat spleen, testis and kidney (Yoshino et al. 1968). The precise mechanism of this induction by iron is not known with certainty. However, following investigations in which ferritin synthesis continued in the presence of a known inhibiter of RNA synthesis (Actinomycin D), it is generally believed to act at the level of translation (Drysdale and Munro 1966, Chu and Fineberg 1969, Millar et al. 1970, Zahringer et al. 1975). Both 'in vivo' and 'in vitro' studies have indicated that apoferritin is synthesised preferentially on free polyribosomes (Hicks et al. 1969, Redman 1969, Puro and Richter 1971). It has recently been suggested (Linder et al. 1974) that free polyribosomes synthesise an apoferritin subunit of molecular weight 13000, whereas membrane-attached polyribosomes synthesise a 19500 molecular weight subunit and that synthesis of the former is preferentially stimulated by iron.

Removal and introduction of iron:

The iron of ferritin is removable by chemical reduction using sodium dithionite (Granick and

Michaelis 1943), ascorbic acid, glutathione, cysteine (Mazur et al. 1955), thioglycollic acid (Crichton and Bryce 1973), reduced flavin nucleotides (Sirivech et al. 1974). Earlier work suggested that 'in vivo' mobilisation of iron was accomplished in a physiological redox system involving xanthine oxidase (Mazur et al. 1958). More recently, however, it has been suggested that an NADH-dependant flavoprotein, "ferriductase", is involved in this process (Osaki and Sirivech 1971, Crichton 1973). "Native apoferritin", prepared by density gradient centrifugation of ferritin preparations, and apoferritin obtained by chemical reduction of ferritin have both been shown to exhibit catalytic activity. Under suitable conditions and in the presence of an oxidising agent the protein oxidises ferrous to ferric iron with the accompanying formation of ferritin (Macara et al. 1972, 1973, Crichton et al. 1973). Niederer (1970) and Harrison et al. (1974) have proposed models for the mechanism of ferritin iron uptake and release. The latter workers have preferred a model in which ferrous iron and small reactants enter freely through channels in the protein shell whereupon crystal growth is initiated at definite sites on the interior of the protein shell. Once this process has commenced, iron is then added directly to the crystallite surface. Iron release takes place from the surface of the iron core microcrystals.

Iron Metabolism in Protein Deficiency

The effects of malnutrition and protein deficiency are reflected in diseases such as kwashiorkor, marasmus and nutritional anaemia which afflict many young children of the underdeveloped countries. These diseases are often compounded by viral and bacterial infection (Scrimshaw 1964) and are not without effect upon iron metabolism. Plasma iron and transferrin levels have been found to be very low in children with kwashiorkor (Lahey et al. 1958, Adams and Scragg 1965, El-Shobaki et al. 1972). Indeed MacFarlane (1969) has suggested that measurement of the serum transferrin level is the most accurate index in the prognosis of kwashiorkor. It has been shown (Morgan 1969, Morgan and Peters 1971) that 10 days of protein depletion in the rat caused a 60-70 per cent fall in the rate of transferrin synthesis. Bethard et al. (1958) investigated the effect of 35 days of protein deprivation on the distribution of an injected tracer dose of radioiron in rat tissues and on erythropoiesis. These workers reported that the protein-deficient animals behaved very differently from the normal fed animals. In the latter 60 per cent of the injected dose accumulated in the bone marrow 3 hours after injection, whereas with the protein-deficient animals very little of the tracer entered the bone marrow for the first 18 hours and a maximum uptake of 37 per cent only occurred after 36 hours. The livers of the protein-

deficient animals contained 5 times the normal amount of radioiron 3 hours after injection and 2.5 times the normal amount 72 hours after injection.

It has been shown that prolonged protein deficiency in the mature male rat results in increased storage of iron in the liver and spleen (Hallgren 1953). Achmed and Ramsay (1974) confirmed these findings and showed that both organs, especially the liver, retained the ability to store the extra iron as ferritin. It might have been envisaged that in times of severe protein deficiency the additional iron would have been deposited preferentially as haemosiderin since this substance contains relatively more iron and less protein than ferritin. Nonetheless, it is well documented that iron stimulates the synthesis of rat liver apoferritin (Drysdale and Munro 1966, Coleman and Matrone 1969, Millar et al. 1970), and Drysdale et al. (1968) observed that when deprived of protein for a period of 4 days the rat was still able to respond normally to the administration of iron. However, the duration of protein deficiency was very much less than in the experiments of Achmed and Ramsay (1974) when the animals were deprived of protein for periods of up to 7 weeks.

The Present Problem

There would appear to be two possible explanations of the finding that prolonged protein deficiency leads

to increased deposition of ferritin iron. The normal course of events might still take place, involving iron stimulated protein synthesis followed by iron uptake, with possible stabilisation of the protein by the presence of iron (Drysdale and Munro 1966). Alternatively, there might be more efficient use of each molecule of protein, with the formation of ferritin molecules of higher average iron content. The operation of the second mechanism would be limited by the theoretical maximum of about 4300 iron atoms per molecule of protein (Fischbach and Anderegg 1965). There would not seem to be any 'a priori' reason why the two mechanisms should not operate simultaneously.

This work was undertaken in an attempt to throw some light on this question. The major experiments done have followed a common pattern. Groups of rats were kept for five weeks on three different diets: 1) normal laboratory diet, 2) a synthetic diet based on that of Bethard et al. (1958) in which the protein was supplied as casein, and 3) an otherwise similar diet in which the casein was replaced by an equicaloric weight of carbohydrate. At the end of the experimental period the total non haem iron and ferritin iron contents of the livers were measured to check the findings of Hallgren (1953) and Achmed and Ramsay (1974). Pure ferritin isolated from the individual livers was assayed for iron and nitrogen. These results were used to calculate the total amount of ferritin protein and the mean number of iron atoms per molecule. Previous studies on iron storage in protein deficiency

have all been performed on male rats. This work has been extended to include females because the rat is one of the species in which, under normal conditions, a sex difference in iron storage is well established (Widdowson and McCance 1948, Kaldor and Powell 1957, Bjorklid and Helgeland 1970, Linder et al. 1973). Bjorklid and Helgeland (1970) studied iron storage up to 16 weeks of age and found that both sexes continued to store iron during this time but the female did so to a greater extent.

Since tissue ferritin molecules do not constitute a homogeneous population, the choice of a method for the isolation of ferritin was of major importance. Even if the existence of isoferritins within a single organ (Drysdale 1970, Urushizaki et al. 1971, Urushizaki et al. 1973) is not taken into account, there is a very real possibility that iron-rich and iron-poor molecules may be differently affected by physical and chemical procedures. One obvious example is high speed centrifugation which has been used for thirty years (Rothen 1944) to separate ferritin molecules of different iron content and upon which is based the isolation procedure of Bjorklid and Helgeland (1970). Mention has also been made (p 7) of the observation of Crichton (1971) that under certain conditions iron-rich ferritin is less susceptible to proteolysis than apoferritin. In the investigation of possible isolation methods it is hardly possible to make experimental evaluation of the relative recoveries of

ferritin molecules over the whole range of composition from zero to 4300 iron atoms per molecule because of the difficulty of establishing base lines in fresh tissue without the use of potentially selective analytical techniques. An isolation procedure, however, has been developed which gives a high overall yield of ferritin iron. The possibility of discrimination against high or low iron molecules is therefore diminished and while there is no direct information on the recovery of apoferritin evidence for its presence in the final product has been obtained.

CHAPTER 2

METHODS

Iron Determinations

All glassware was rendered free from contaminating iron by an overnight soak in hydrochloric acid (1 mol/ℓ). "Aristar" Ammonium Sulphate (BDH, Poole, Dorset) was used, all other reagents were of analytical grade.

All iron determinations were made by the 2,2´-bipyridyl method (Hill 1931). Iron concentrations were calculated from a standard calibration curve (Fig.2.1) obtained using ferric ammonium sulphate.

Reagents:

Hydrochloric acid (1 mol/ℓ)

Sodium Sulphite (1.5 mol/ℓ)

2,2´-bipyridyl (0.5% w/v) in acetic acid (60% v/v)

Procedure:

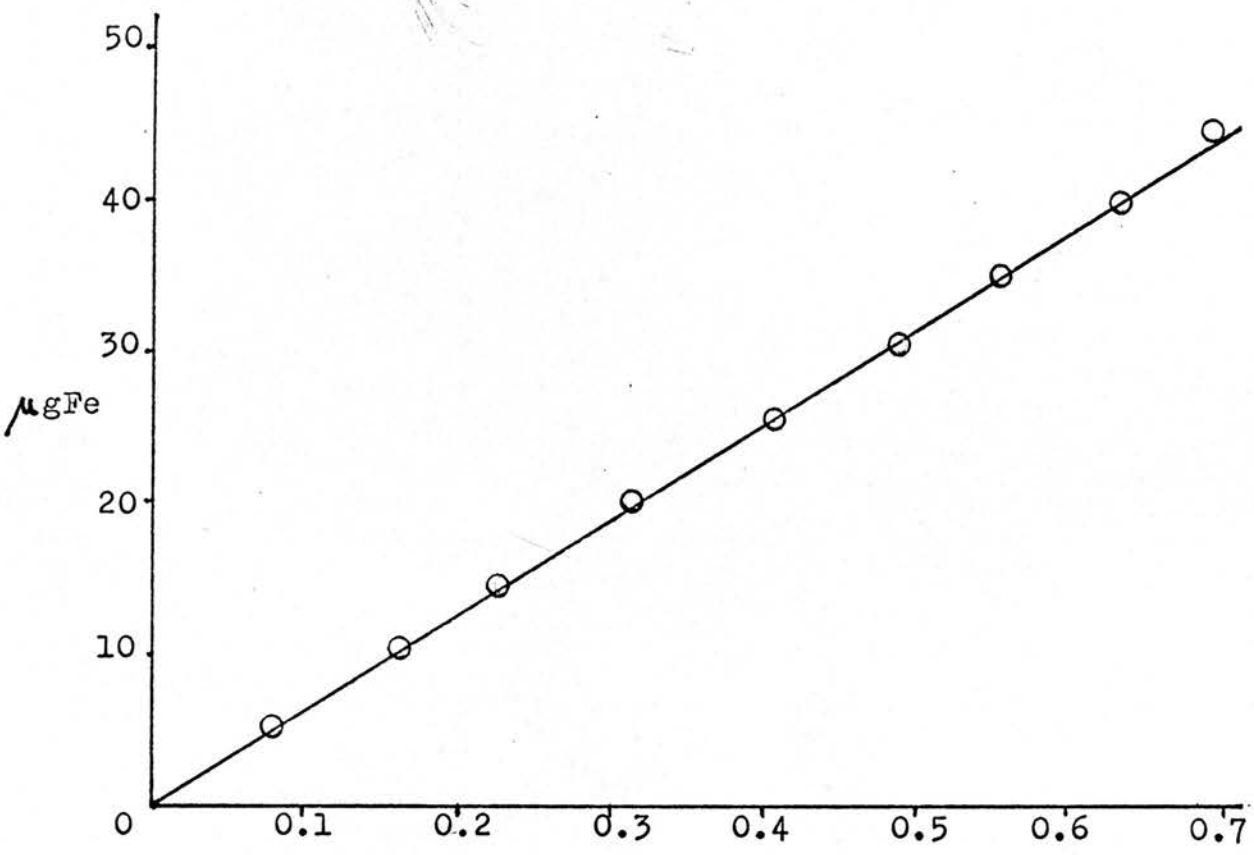
A solution of ferric ammonium sulphate (100μg Fe/ml) in hydrochloric acid (0.01 mol/ℓ) is treated with 0.8 ml hydrochloric acid, 1.0 ml sodium sulphite and heated in a boiling water bath (5 mins). 2,2´-bipyridyl (0.5ml) is added and the volume adjusted to 10 ml with water and the heating continued for a further 5 minutes. After cooling the volume is readjusted to 10 ml with water and the absorbance of the pink ferrous-bipyridyl complex is measured at 520 nm in a Unicam SP 1800 Spectrophotometer.

1. Total non haem iron (TNHI) determination

Much of the iron in most fresh tissues is present in the form of haemoglobin in contaminating blood. The iron

FIG. 2.1

Iron Calibration Curve



Absorbance 520 nm

A solution of ferric ammonium sulphate (in hydrochloric acid 0.01 mol/ℓ) was used as standard.
Iron was measured after formation of the ferrous-dipyridyl complex in a final volume of 10 ml (p.15).

in the storage compounds ferritin and haemosiderin together makes up almost the total amount of other iron present (Drysdale and Ramsay 1965). In the gross quantitative context the traces present in cytochromes, iron-sulphur proteins and the like can be neglected (Drabkin 1951). To measure TNHI it is, therefore, necessary to separate haemoglobin from ferritin and haemosiderin or to employ a procedure for determination of iron which is not affected by the presence of haemoglobin. The method actually used (Drysdale and Ramsay 1965) makes use of both principles. It takes advantage of the fact that rat haemoglobin is soluble in half saturated ammonium sulphate while ferritin and haemosiderin are both precipitated along with other tissue proteins. Iron is then determined in the precipitate by a method which leaves the iron of any residual haemoglobin almost unaffected. Ferritin and haemosiderin are decomposed by heating with dilute hydrochloric acid and the pink ferrous 2,2́-bipyridyl complex is formed in the presence of sodium sulphite.

Reagents:

As for Fe determination (p.15)

Procedure:

Fresh frozen liver is homogenised for 60 seconds with four times its weight of water in a top-drive homogeniser (MSE). The homogenate (1 or 2 ml) is treated with an equal volume of saturated ammonium sulphate in a test tube graduated at 10 ml and left at 4 C for a minimum of 2 hours. The precipitate is recovered by centrifugation

(1400g, 15 mins) and the supernatant discarded. The precipitate is suspended in 5 ml water, 0.8 ml hydrochloric acid added and the tube is heated in a boiling water bath (15 mins). 2,2'-bipyridyl (0.5 ml) and sodium sulphite (1.0 ml) are added, the volume is adjusted to 10 ml with water and the heating continued for a further 30 minutes. After cooling, the volume is readjusted to 10 ml and the tube is centrifuged to remove any turbidity after vigorous shaking with chloroform (1 ml). The absorbance of the pink ferrous-bipyridyl complex is measured at 520 nm in a Unicam SP 1800 Spectrophotometer. Iron concentrations are obtained from a standard calibration curve (Fig.2.1).

The procedure has been tested by comparison with two other methods. In the first of these, precipitation with ammonium sulphate was performed in the same way. The precipitate was then suspended in sodium hydrogen carbonate (0.1 mol/l) and the ferritin and haemosiderin were freed from the remaining traces of haemoglobin by re-precipitation. The precipitate was again suspended, aliquots were taken for iron analysis as described (p.16) and also for iron analysis after destruction of the organic matter by heating with concentrated sulphuric acid (0.2 ml), concentrated nitric acid (0.2 ml) and perchloric acid (0.1 ml).

In the second comparison, ferritin, haemosiderin and haemoglobin were separated from one another on a column of CM-cellulose (Drysdale and Ramsay 1965). In this study

acetate buffers were employed instead of citrate and ferritin was quickly eluted from the column with acetate buffer, (0.1 mol/ℓ) pH 5.5.

Comparative results are given in Table 2.1 and Fig. 2.2. Fig. 2.2 confirms that the iron estimation, which is well established in simple aqueous solution (Hill 1931), is unaffected by tissue constituents in the ammonium sulphate precipitate. Additional figures obtained by the use of different quantities of hydrochloric acid in the pretreatment of the suspended precipitate showed that the quantity used in the procedure is adequate. Table 2.1 shows, if the validation of the CM-cellulose separation on this small scale by Drysdale and Ramsay (1965) is accepted, that the ammonium sulphate treatment used precipitates all the ferritin and haemosiderin present. For these reasons it appeared satisfactory to use the procedure described on p.16.

2. Ferritin iron determination

In order to select a satisfactory routine method for the measurement of ferritin iron in homogenised tissues, 3 procedures were compared (Fig. 2.3). These were based on the separation of ferritin from haemosiderin by (i) centrifugation at pH 4.8, (ii) heat coagulation at 70 C, (iii) CM-cellulose chromatography. Methods (i) and (ii) were compared and in each procedure ferritin iron was determined by the bipyridyl method (p.16) on the precipitates obtained following a) precipitation with half saturated

TABLE 2.1

Tissue Total Non Haem Iron Determination - A Comparison between CM-Cellulose Chromatography and $(NH_4)_2SO_4$ Precipitation

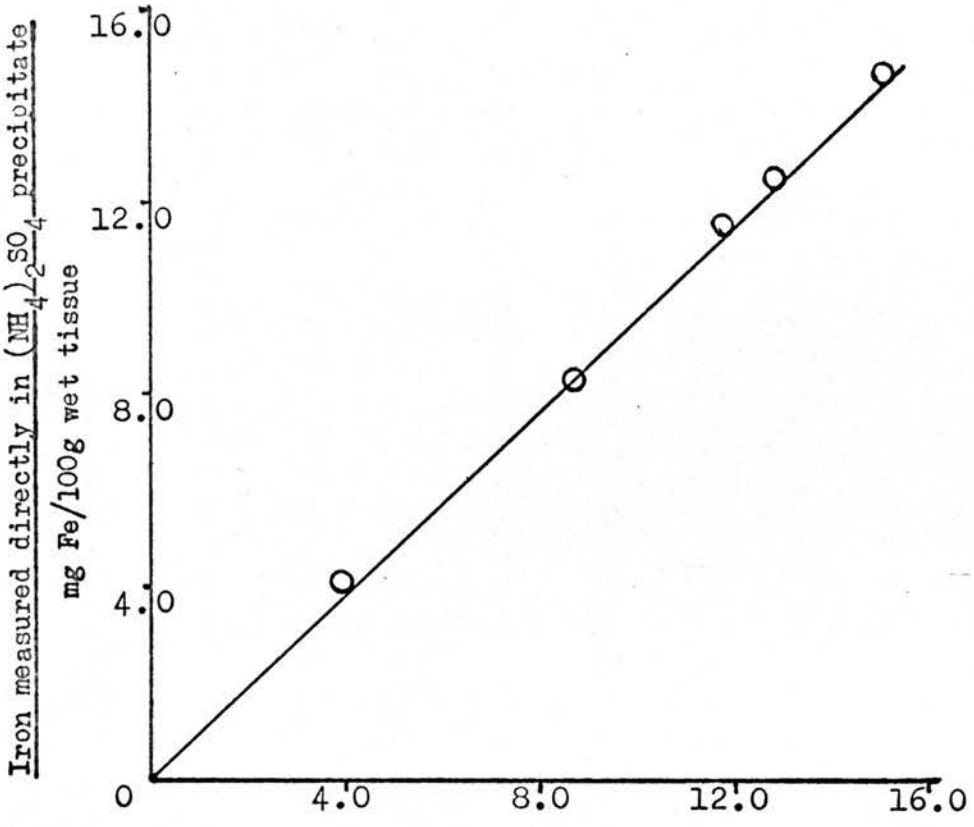
Experiment	$(NH_4)_2SO_4$ Precipitation	CM-Cellulose Chromatography	
1	12.5	Ferritin	9.7
		Haemosiderin	3.4
		Total	13.1
2	13.0	Ferritin	9.7
		Haemosiderin	3.4
		Total	13.1
3	17.0	Ferritin	13.2
		Haemosiderin	3.3
		Total	16.5

All quoted figures are the means of duplicates.
A crude tissue homogenate was prepared (p.16). An aliquot was chromatographed on CM-Cellulose (Drysdale and Ramsay 1965) and iron (mg/100g wet tissue) was measured in the ferritin and haemosiderin fractions after formation of the ferrous dipyridyl complex (p.17). A similar aliquot was precipitated by 50 per cent saturation with $(NH_4)_2SO_4$ and the precipitate was assayed for iron (mg/100g wet tissue).

FIG. 2.2

Tissue Total Non Haem Iron Determination -

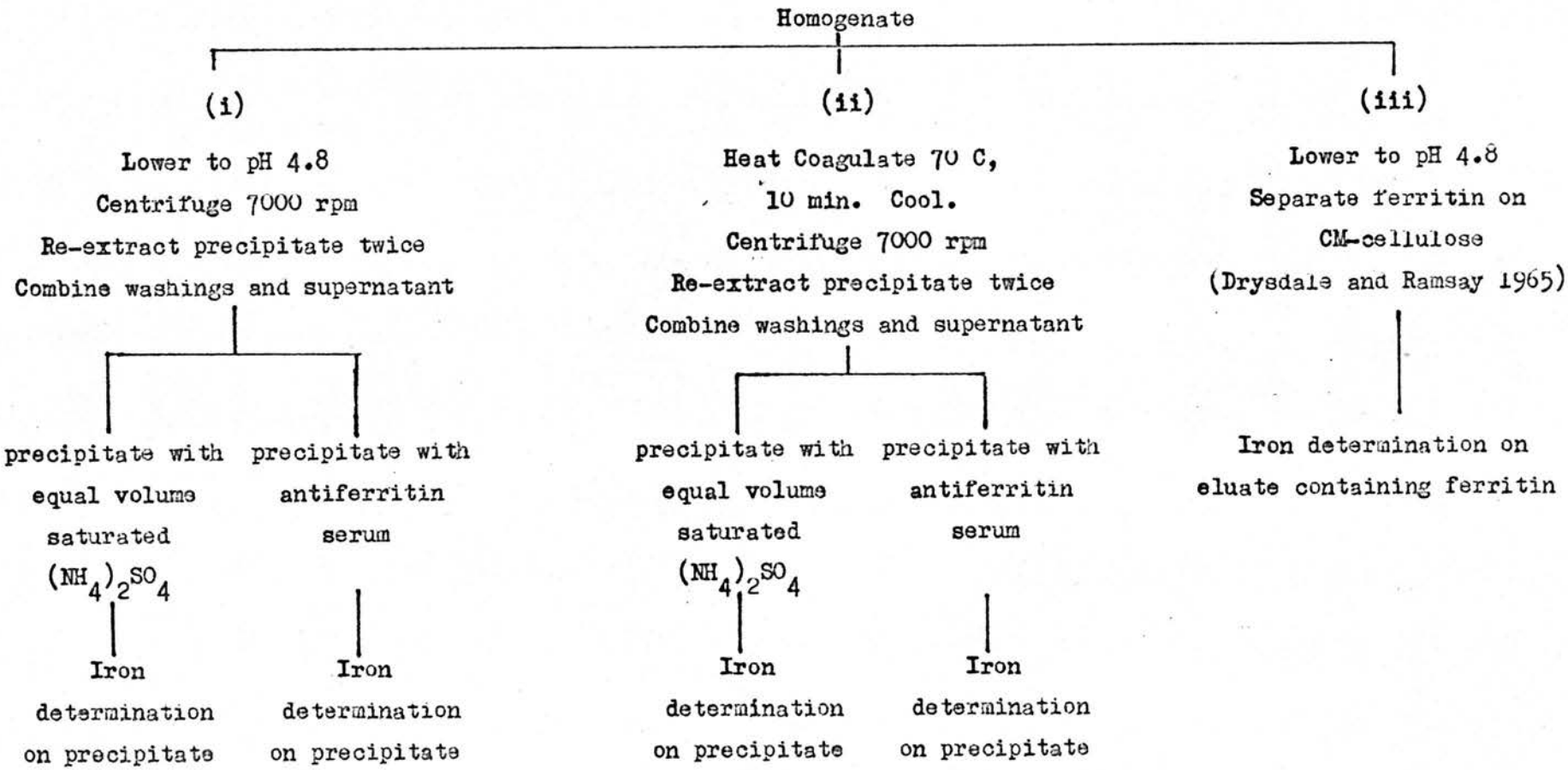
A Comparison of Procedures



A crude tissue homogenate was twice precipitated by 50 per cent saturation with $(NH_4)_2SO_4$ and the precipitate was finally suspended in $NaHCO_3$. Aliquots of this suspension were precipitated by 50 per cent saturation with $(NH_4)_2SO_4$. Iron (mg/100g wet tissue) was measured as the ferrous dipyridyl complex 1) directly in the precipitate and 2) in the digest following wet ashing of the precipitate. The line drawn is the 45^{o} line for perfect correspondence between the two methods.

FIG. 2.3

An Outline of Procedures used for Ferritin Iron Determination



ammonium sulphate, b) precipitation with antiferritin serum. Method (i) was also compared with (iii), but in this instance precipitation with antiferritin serum was not performed. The procedure finally adopted depended on the observation (Drysdale and Ramsay 1965) that centrifugation of a tissue homogenate, after suitable adjustment of ionic strength and pH, removed haemosiderin completely.

(i) Ferritin iron determination on centrifuged homogenate (pH 4.8)

Reagents:

Acetic acid (0.5 mol/ℓ)

Acetate buffer (0.025 mol/ℓ) pH 4.8

Reagents for Fe determination (p.15)

Procedure:

The tissue homogenate (p.16) is lowered to pH 4.8 with acetic acid and centrifuged at 7000 rpm (MSE 18 Centrifuge) for 20 minutes. The precipitate is extracted 2 times with buffer (10 ml) and centrifuged at 7000 rpm (15 mins) after each washing. The supernatant and the washings are combined and diluted to twice the original volume of the homogenate. Suitable aliquots are taken and analysed by two separate procedures: a) measurement of non haem iron in the precipitate obtained by half saturation with ammonium sulphate (p.16) and b) measurement of iron in the precipitate obtained by treatment with antiferritin serum (p.21).

(ii) Ferritin iron determination in the heat supernatant

The second procedure tested relied on the widely used removal of haemosiderin by heat coagulation. It is common to centrifuge the homogenate after heating to a temperature between 65-80 C and to take an aliquot from the supernatant for iron analysis. Many workers calculate the results on the assumption that the proportional volume of the non aqueous material in the coagulum can be ignored, but Linder and Munro (1972) have found that this assumption leads to high results. Preliminary experiments in this laboratory have confirmed their conclusion and the heat coagulum produced by heating to 70 C for 10 minutes has, therefore, been repeatedly extracted to remove the ferritin completely, so that the erroneous assumption is avoided.

Procedure:

A 1-in-5 homogenate of liver in water (20 ml) is heated, with constant agitation, for 10 minutes at 70 C. After cooling in an ice bath (15 mins) the coagulum is removed by centrifugation at 7000 rpm (MSE 18 Centrifuge). The precipitate is extracted twice with phosphate (0.01 mol/ℓ) - sodium chloride (0.04 mol/ℓ) buffer, pH 7.0 (7.0 ml). The washings and supernatant are combined and diluted to twice the original volume (40 ml). Suitable aliquots are analysed by a) measurement of non haem iron in the precipitate obtained by half saturation with ammonium

sulphate (p.16) and b) measurement of iron in the precipitate obtained after treatment with antiferritin serum (p. 21).

(iii) Ferritin iron determination by CM-cellulose chromatography

The third procedure used is the chromatographic separation of tissue iron components on CM-cellulose (Drysdale and Ramsay 1965) as previously mentioned (p.17). For the present purpose only the eluate containing ferritin was analysed.

(iv) Immunoprecipitation of ferritin

Precipitation of ferritin from crude tissue extracts by treatment with antiferritin serum has often been employed (Mazur and Shorr 1950) in the assay of ferritin protein and ferritin iron. In this work the precipitin reaction has been used to check methods for the separation of ferritin from other compounds containing iron. The serum used was an antiserum to horse ferritin, raised in rabbits and kindly donated by Dr. J.D. Cook, Department of Medicine, University of Washington, Seattle, U.S.A. Its preparation as described by Dr. Cook is given in Appendix I.

The potency of the antiserum preparation was established in this laboratory by serial dilution. Although the limit was not determined, 1 ml of a 1-in-10 dilution of the antiserum in borate buffer (0.15 mol/ℓ) pH 8.8 was found to satisfactorily precipitate up to 35μg of ferritin iron. A 1-in-5 dilution of the antiserum has been routinely employed.

Procedure:

The conditions for the precipitin reaction were those described by Mazur and Shorr (1950) and Linder-Horowitz et al. (1970). The antigen solution (1.0 or 2.0 ml), to which sodium chloride was added to give a total salt concentration of 0.15 mol/ℓ, was added to 1 ml of the antiserum. When necessary borate buffer (0.15 mol/ℓ, pH 8.8) was added to give a final volume of 3 ml. The tubes were kept for one hour at 37 C and a further 18 hours at 5 C. The precipitate was recovered by centrifugation (1400g, 15 mins) and washed twice with 3 ml portions of sodium chloride solution (0.15 mol/ℓ). Iron determinations were carried out on the precipitate as previously described (p.17).

The data of Table 2.2 which compares the pH 4.8 extraction with CM-cellulose (correlation coefficient r = 0.997) confirm that the former removes haemosiderin completely. This is further supported in Table 2.3 by the results obtained by immunoprecipitation in which only ferritin iron was measured in the antigen-antibody complex. The results of Table 2.3 also demonstrate a satisfactory agreement between the different procedures. As noted previously (p.19) the procedure adopted for all routine analyses was that in which ferritin iron in the supernatant obtained after centrifugation at pH 4.8 (p.19) was measured following precipitation with ammonium sulphate.

TABLE 2.2

Ferritin Iron Determination - A Comparison between CM-Cellulose Chromatography and Ammonium Sulphate Precipitation

Experiment	CM-Cellulose Chromatography	$(NH_4)_2SO_4$ Precipitation of pH 4.8 Supernatant
1	7.2	7.0
2	8.5	8.8
3	9.8	9.8
4	10.5	10.2
5	12.2	12.0
6	13.0	12.5
7	13.8	14.5
8	24.0	25.5

Correlation Coefficient r = 0.997

All quoted figures are the means of duplicates.
An aliquot of a crude tissue homogenate was chromatographed on CM-Cellulose (Drysdale and Ramsay 1965) and only the ferritin containing fractions were assayed for iron (mg/100g wet tissue). The "pH 4.8 supernatant" (p.19) prepared from the crude tissue homogenate was precipitated by 50 per cent saturation with $(NH_4)_2SO_4$ and the iron (mg/100g wet tissue) in the precipitate determined.

TABLE 2.3

Ferritin Iron Determination - A Comparison of Procedures

Experiment	Heat Supernatant		pH 4.8 Supernatant	
	$(NH_4)_2SO_4$ Precipitation	Immuno-Precipitation	$(NH_4)_2SO_4$ Precipitation	Immuno-Precipitation
1	17.0	16.4	16.7	16.5
2	12.5	12.4	12.3	13.0
3	6.7	6.5	6.9	6.7
4	6.6	6.5	6.4	6.4
5	6.6	6.6	6.3	6.4

All quoted figures are the means of duplicates.
A "heat supernatant" (p.20) and a "pH 4.8 supernatant" (p.19) were prepared from the same crude tissue homogenate. In each case the ferritin was precipitated from the supernatant by 1) 50 per cent saturation with $(NH_4)_2SO_4$; 2) precipitation with ferritin antibody (p.22). Iron (mg/100g wet tissue) was determined in both the immunoprecipitate and the $(NH_4)_2SO_4$ precipitate after formation of the ferrous dipyridyl complex (p.17).

Protein Determination

The principal methods for the analysis of both purified protein and tissue protein have been comprehensively reviewed by Munro and Fleck (1969). Included among these are nitrogen determination, biuret reaction, phenol-biuret reaction, ultra violet absorption. In the analysis of ferritin the use of ultra violet absorption is precluded by the presence of the iron micelle which itself exhibits intense absorption in the desired region 240-280 nm. The phenol-biuret procedure (Lowry et al. 1951) has been reported to give inconsistent results for ferritin determination (Crichton 1969) but the reason for this was not given. There are also difficulties about the selection of a suitable standard. Linder and Munro (1972) have reported that horse spleen apoferritin gives 90 $\pm$ 3% of the colour of bovine serum albumin whereas purified rat liver apoferritin gives 140 $\pm$ 5% of the colour generated by a similar weight of albumin. The procedure chosen for this investigation was, therefore, the fundamental method of nitrogen determination by the micro-Kjeldahl technique. Crichton (1969) has also adopted this solution to the problem. Nitrogen values have been multiplied by a factor of 6.25 for conversion into protein. The nitrogen content of horse spleen ferritin has been estimated to be 16.1-16.3% (Harrison 1964).

The optimal temperature for digestion in open tubes is 360-410 C. Fleck (1967) has successfully used a digestion temperature of 370 C, obtained by the addition of 0.8g potassium sulphate per ml of concentrated sulphuric acid, in conjunction with a mercury catalyst. These conditions have been adopted for this work. Ammonia was liberated from the mercury-ammonia complex in a Conway diffusion unit (Conway 1947) as described by Weil-Malherbe and Green (1955) and estimated titrimetrically using hydrochloric acid.

Reagents:

Kjeldahl mercury catalyst tablet containing potassium sulphate (B.D.H., Poole, Dorset)

'Aristar' concentrated sulphuric acid

Hydrochloric acid 0.01 mol/ℓ

Mixed Indicator:

0.33% w/v Bromocresol Green
0.66% w/v Methyl Red
} in Ethanol

Alkali: 60% w/v potassium hydroxide containing 10% w/v sodium thiosulphate

1% w/v Boric Acid Solution (adequate for up to 300μg Ammonia nitrogen) prepared as follows:

Boric acid (5g) is dissolved in ethanol (100 ml) and water (350 ml) and transferred to a 500 ml volumetric flask. Mixed indicator (5 ml) is added and a faint reddish end-point is obtained by the addition of a few drops of sodium

hydroxide(0.01 mol/ℓ). The mixture is then made up to the mark with water.

Procedure:

Protein solution (1.0 or 2.0 ml), catalyst tablet (200 mg) and sulphuric acid (0.25 ml) are measured into a micro-Kjeldahl flask (bulb capacity 4-4.5 ml) calibrated at 5.0 ml. The excess water is carefully boiled off and the flask is transferred to a digestion rack and digested for a further 45 minutes. On cooling, the solid digest is dissolved and made up to a 5.0 ml volume with water.

The central well of the Conway number 1 diffusion unit contained the boric acid solution (1.5 ml). An aliquot of the digest (1.0 or 2.0 ml) and the alkali (1.0 ml) are allowed to react in the outer chamber for 4 hours at 20-25 C. Effective sealing of the apparatus is achieved using a gum acacia solution (Conway 1947). The liberated ammonia which is trapped in the boric acid solution is titrated with hydrochloric acid to the same end-point as the blank. The latter was prepared by digesting only the catalyst and sulphuric acid. Throughout the work standard solutions of ammonium sulphate have been estimated and overall recoveries from the combined digestion and diffusion have been in excess of 95 per cent. Table2.4 presents some representative recoveries obtained with ammonium sulphate, leucine and alanine.

TABLE 2.4

Nitrogen Recovery following Micro-Kjeldahl Digestion Diffusion in Conway Unit

Substance	Per Cent Nitrogen Recovered Replicate		
	I	II	III
Ammonium Sulphate	96.7	100.4	98.2
Alanine	97.4	98.0	-
Leucine	98.2	96.6	-

Standard solutions (100 µg N/ml) of ammonium sulphate, alanine and leucine were digested as described (p.25). The ammonia liberated in the Conway diffusion unit was estimated titrimetrically with hydrochloric acid. Each figure is the mean of two determinations.

Calculation of Ferritin Protein

The procedure used by Drysdale and Munro (1965) has been employed in the calculation of total ferritin protein per liver. The ferritin iron content of the tissue homogenate (p.19) is initially determined and the iron to protein ratio of the purified ferritin is measured. With a knowledge of these two measurements, the ferritin protein present in the original liver homogenate is readily calculated.

The number of iron atoms per molecule of protein has been calculated from the iron to nitrogen ratio, assuming molecular weight of 440,000 for apoferritin (Bjork and Fish 1971), although a theoretical value of 444,000 is obtained when a molecular weight of 18,500 for each of the 24 subunits is used in the calculation. The difference between the values obtained by using the different molecular weights (0.9%) is probably much less than the total experimental error.

Gel Electrophoresis

Polyacrylamide gel electrophoresis has been employed as a criterion of the homogeneity of ferritin preparations. A gel concentration of 5 per cent containing acrylamide: Methylene-bis-acrylamide in the ratio 97.5:2.5 by weight has been chemically polymerised with ammonium persulphate and N,N,N′,N′-tetramethylethylenediamine (Davies 1964). After pre-running the gels for 30 minutes in the running

buffer electrophoresis has been routinely performed at pH 8.5 (tris-citrate buffer 0.05 mol/ℓ) and 3 mA per tube. Gels were stained for protein with Naphthalene Black (0.1% w/v) in acetic acid (7% v/v) for 1 hour and were destained with acetic acid (7% v/v). The Prussian Blue reaction was used to stain for iron using potassium ferrocyanide (2% w/v) in hydrochloric acid (2% v/v) (Linder-Horowitz et al. 1970). Gels stained for protein were scanned at 580 nm (Unicam SP 500 with Gilford Scanner attachment) after prior treatment in glacial acetic acid/methanol/water (7:35:58).

Electrophoresis has also been performed at pH 7.9 (tris-citrate 0.05 mol/ℓ) (Drysdale and Munro 1965) and at pH 9.3 (tris-glycine 0.05 mol/ℓ) (Linder-Horowitz et al. 1970). At the higher pH a very sharp, rapidly moving band in addition to the major protein band was observed on staining with Naphthalene Black. Identical samples did not yield this band when electrophoresis was undertaken at either pH 7.9 or pH 8.5. A minimum pre-run period of 2 hours for the gels at pH 9.3, however, eliminated the additional band. After much investigation it was concluded that the band was an artefact of the buffer system. It has been reported (Brewer 1967, Fantes and Ferminger 1967) that when persulphate was used in the polymerisation, artefacts have been produced when proteins were run on the gels. In a report by Crichton et al. (1973), an illustration of the electrophoresis of ferritin showed a rapid-moving band similar to the one observed in this work at pH 9.3 which the author dismissed as an artefact without discussion.

The Isolation of Ferritin

The original procedure (Laufberger 1937, Granick 1946) took advantage of the heat stability of ferritin to remove considerable quantities of unwanted material from the tissue homogenate by heating at 80 C. The coagulated proteins were removed and the ferritin in the supernatant was further purified by ammonium sulphate precipitation and recrystallisation from cadmium sulphate. Many investigators have since employed heat coagulation as an initial step and have subsequently purified the protein by a combination of various physico-chemical techniques such as ion exchange chromatography, ammonium sulphate precipitation, differential centrifugation and gel filtration chromatography. In some studies ferritin has been isolated from the heat supernatant by immunoprecipitation with a ferritin antibody. Although many of the procedures used have produced electrophoretically homogeneous ferritin preparations, critical attention to the overall yield has not always been made.

The original procedure, while suited to large scale isolations, was inefficient in terms of final yield (Granick 1943, Fineberg and Greenberg 1955). Fineberg and Greenberg (1955) observed that the conditions for recrystallisation of guinea pig ferritin differed from those for horse spleen and that under optimal conditions the yield was 30 per cent. For quantitative estimations, they pre-

cipitated ferritin from a heat supernatant (75 C, 5 min) using a ferritin antibody. Others have used this immunoprecipitation technique (Mazur and Shorr 1950, Mazur et al. 1960, Yoshino et al. 1968, Bernier et al. 1970). This method has been criticised (Linder and Munro 1972) on the grounds that non specific co-precipitation of contaminating proteins occurred and this led to erroneously high estimates of ferritin protein. In an attempt to partially purify ferritin prior to immunoprecipitation Yu and Fineberg (1965) tried ion-exchange chromatography. CM-cellulose proved unsuitable and resulted in excessive losses of ferritin; DEAE-cellulose was more satisfactory but considerable losses of protein were again observed. Better recoveries, however, were obtained when heat coagulation was performed at a lower temperature (65 C). These workers concluded that rat liver ferritin was more susceptible to heat denaturation than horse spleen ferritin. Suran and Tarver (1965) have reported the complex behaviour of ferritin on DEAE-cellulose. Ferritin, purified by a procedure which avoided heat coagulation, was observed to fractionate in 3 components on chromatography. Penders et al. (1968) have reported the fractionation of rabbit liver ferritin on DEAE-cellulose. Porter (1974) has prepared ferritin from human spleen by a method in which heat coagulation was avoided; in this instance both CM-cellulose and DEAE-cellulose were employed. This latter medium has been employed in the isolation of rabbit

liver and spleen ferritin (Muraoka et al. 1966) and tuna fish spleen ferritin (Kato et al. 1966). Drysdale and Munro (1965) chromatographed a heat supernatant (80 C) on CM-cellulose and further purified ferritin by ammonium sulphate precipitation and Sephadex G-200 chromatography. This procedure has been followed by others (Coleman and Matrone 1969, Ove et al. 1972, Niitsu 1973, Urushizaki et al. 1973, Millar et al. 1970, Shafritz et al. 1973) although the conditions for heat coagulation have sometimes been altered; for example, Coleman and Matrone (1969) heated the tissue homogenate at 75-78 C for 20-30 minutes, whereas Shafritz et al. (1973) heated at 75 C for 2 minutes.

Chromatography on CM-cellulose has been reported to cause considerable losses of ferritin (Yu and Fineberg 1965, Linder and Munro 1972) but Drysdale and Ramsay (1965) described an analytical scale quantitative fractionation of tissue iron compounds on this medium. Alpert et al. (1973), Powell et al. (1974) have preferred to omit CM-cellulose chromatography from their isolation procedure to prevent fractionation of isoferritins. An electrophoretically homogeneous product was reported after heat coagulation (75 C, 2 min), ammonium sulphate precipitation and Sephadex G-200 chromatography (Alpert et al. 1973). Urushizaki et al. (1973) have described the separation of pure rat liver ferritin into 3 components on CM-cellulose.

Electrophoretically homogeneous rabbit liver ferritin has been prepared in 70 per cent yield (Penders et al. 1969) using a differential ultracentrifugation technique.

Bjorklid and Helgeland (1970) modified the procedure for the preparation of rat liver ferritin: the heat supernatant (72-74 C, 10 min) was first precipitated with ammonium sulphate before the differential ultracentrifugation was undertaken. Crichton et al. (1973) used differential ultracentrifugation in the isolation of human liver and spleen ferritin.

The procedure used in this work was devised with two goals in view: a) maximization of yield b) adaptation of separation procedures so that no one step would be likely to fractionate ferritins of slightly different structure and properties.

1. Preliminary investigations into heat coagulation and CM-cellulose chromatography

Preliminary experiments in this laboratory have confirmed the finding of Drysdale and Munro (1965) that substantial purification of the heat supernatant (80 C) of a crude tissue homogenate was effected by CM-cellulose chromatography. However, careful control of the temperature during heat coagulation as advocated by Yu and Fineberg (1965) suggested that overheating might entail a risk of denaturing ferritin to such an extent as to render it unelutable from an ion exchange column. Further investigation has confirmed this suspicion. Moreover, it was demonstrated that ferritin was precipitated, presumably non-specifically with denatured protein of other kinds,

when the heat supernatant was lowered to pH 4.8 prior to CM-cellulose chromatography.

A liver homogenate (15 ml, p.16) was heated in a 25 ml conical flask (5-15 mins). After cooling in ice (15 mins) the coagulum was removed by centrifugation at 1400g (15 mins). Aliquots (1.5 ml) of the supernatant were treated with an equal volume of saturated ammonium sulphate and the resulting precipitate was assayed for iron. The remainder of the supernatant was lowered to pH 4.8 with acetic acid (0.5 mol/l). The resulting precipitate was removed by centrifugation. Aliquots (1.5 ml) of the supernatant were assayed for iron and were also chromatographed on CM-cellulose. The ferritin containing eluate was ultimately assayed for iron.

It is clear that after heating to 80 C, both acidification to pH 4.8 and subsequent chromatography on CM-cellulose caused losses of iron (Table 2.5). At 70 C, (Table 2.6), smaller losses of iron are clearly due to loss of ferritin since all the iron measured in the heat supernatant is immunoprecipitable (p.22).

In view of these findings, attempts were made to purify ferritin by a procedure which avoided the use of heat coagulation. CM-cellulose chromatography of a homogenate adjusted to pH 4.8, from which cellular debris and other precipitated material including haemosiderin had been removed by centrifugation, followed by ammonium sulphate precipitation and Sephadex G-200 chromatography

TABLE 2.5

Heat Coagulation at 80 C - Ferritin Iron Assay

Experiment	Time of Heating (min)	Heat Supernatant	Heat Supernatant pH 4.8	CM-Cellulose Chromatography
1	10	4.0	0.7 (18)	0.7 (100)
2	10	15.7	3.7 (24)	2.0 (55)
3	15	13.7	7.8 (57)	3.1 (40)
	10	13.5	9.3 (69)	8.8 (95)
4	5	23.6	22.0 (93)	22.0 (100)
	15	23.6	20.1 (89)	18.9 (94)
5	10	23.8	17.6 (74)	16.3 (93)

The heat supernatant was prepared from the crude tissue homogenate. Suitable aliquots of the heat supernatant were precipitated with $(NH_4)_2SO_4$ (50 per cent saturation) and iron (mg/100g wet tissue) was measured in the precipitate. The remainder of the heat supernatant was lowered to pH 4.8 and aliquots were taken for 1) precipitation with $(NH_4)_2SO_4$ (50 per cent saturation) in which iron (mg/100g wet tissue) was assayed in the precipitate 2) CM-cellulose chromatography where the ferritin containing eluate was assayed for iron (mg/100g wet tissue). The figures in parentheses indicate per centage iron recovery following 1) pH adjustment and 2) chromatography.

TABLE 2.6

Heat Coagulation at 70 C - Ferritin Iron Assay

Experiment	Time of Heating (min)	Heat Supernatant	Heat Supernatant pH 4.8	CM-Cellulose Chromatography
1	10	5.8	5.4 (93)	3.2 (59)
2	10	5.8	5.3 (91)	4.2 (79)
3	10	26.8	26.5 (99)	23.3 (87)
4	10	4.4	3.7 (84)	3.5 (95)
5	10	5.3	5.2 (98)	4.4 (85)
6	15	11.8	11.2 (95)	7.5 (67)
7	15	14.0	13.3 (95)	8.8 (66)
8	15	18.0	17.6 (98)	10.6 (60)
9	15	23.5	23.3 (99)	20.5 (88)
10	15	5.7	4.6 (80)	3.7 (80)

The heat supernatant was prepared from the crude tissue homogenate. Aliquots of the heat supernatant were precipitated with $(NH_4)_2SO_4$ (50 per cent saturation) and iron (mg/100g wet tissue) was measured in the precipitate. The remainder of the heat supernatant was lowered to pH 4.8 and aliquots were taken for 1) precipitation with $(NH_4)_2SO_4$ and iron (mg/100g wet tissue) was measured in the precipitate 2) CM-cellulose chromatography where the ferritin containing eluate was assayed for iron (mg/100g wet tissue). The figures in parentheses indicate per centage iron recovery following 1) pH adjustment 2) chromatography.

gave high yields in terms of iron but failed to achieve an electrophoretically pure product. Further work, however, demonstrated that heating the CM-cellulose eluate at pH 5.5 for 5 minutes at 70 C removed an appreciable quantity of coagulable impurity and the final product when this latter treatment was included, was electrophoretically homogeneous.

2. Procedure for isolation of ferritin

Extraction of ferritin from the tissue homogenate:

A homogenate of 1 part liver plus 4 parts water (p.16) is brought to pH 4.8 with 0.5 mol/ℓ acetic acid. This is then centrifuged at 7000 rpm (MSE 18 Centrifuge) 5 C for 20 minutes. The precipitate is washed 2 times with acetate buffer (0.025 mol/ℓ) pH 4.8 (10.0 ml), centrifuging at 7000 rpm for 15 minutes after each washing. The supernatant and the washings are combined and diluted to twice the original volume of the homogenate. Suitable aliquots (1-2 ml) are taken for ferritin iron assay (p.19), the remainder is chromatographed on CM-cellulose.

CM-cellulose chromatography at 5 C:

A column of CM-cellulose (bed volume 2.2 cm diameter x 4.0 cm length) is equilibrated with acetate buffer (0.025 mol/ℓ) pH 4.8. A maximum of 40 ml of the pH 4.8 supernatant is applied to the column and elution with pH 4.8 buffer is continued until the turbidity of the eluate is cleared. Immediately upon clearance acetate buffer (0.1 mol/ℓ) pH 5.5 is passed until all the ferritin which moves down the

column as a brown band is eluted (25-35 ml). The total time for chromatography was approximately 90 minutes.

Heat coagulation at 70 C:

The ferritin eluate from above is heated at 70 C for 5 minutes. After cooling, the precipitated impurities are removed by centrifugation at 7000 rpm for 15 minutes.

Precipitation with ammonium sulphate:

Ferritin is precipitated, 5 C for 18 hours, from the heat supernatant by ammonium sulphate added as solid in sufficient quantity to give a final saturation of 60 per cent (36.1g/100 ml). This method is preferred to precipitation by the addition of an equal volume of saturated ammonium sulphate because in the latter instance an occasional low recovery (80 per cent) was observed. In tissue analyses precipitation by 50 per cent saturation with ammonium sulphate has been shown to be adequate (p.18) suggesting that the co-precipitation of other proteins in the crude tissue preparation most probably aids the precipitation of ferritin. The precipitated protein is recovered by centrifugation at 7000 rpm for 30 minutes and the precipitate is dissolved in 2.0 ml phosphate buffer (0.02 mol/ℓ) pH 7.0. This solution is chromatographed on Sephadex G-200.

Sephadex G-200 chromatography:

The sample (2-3 ml) is applied to a column (1 cm^2 x 60 cm) previously equilibrated with phosphate buffer (0.02 mol/ℓ) pH 7.0; this also serves as the eluting buffer. The flow rate is adjusted to about 7 ml per hour and the

iron containing fractions (1.0 ml) are collected. The ferritin is usually collected in 10 fractions.

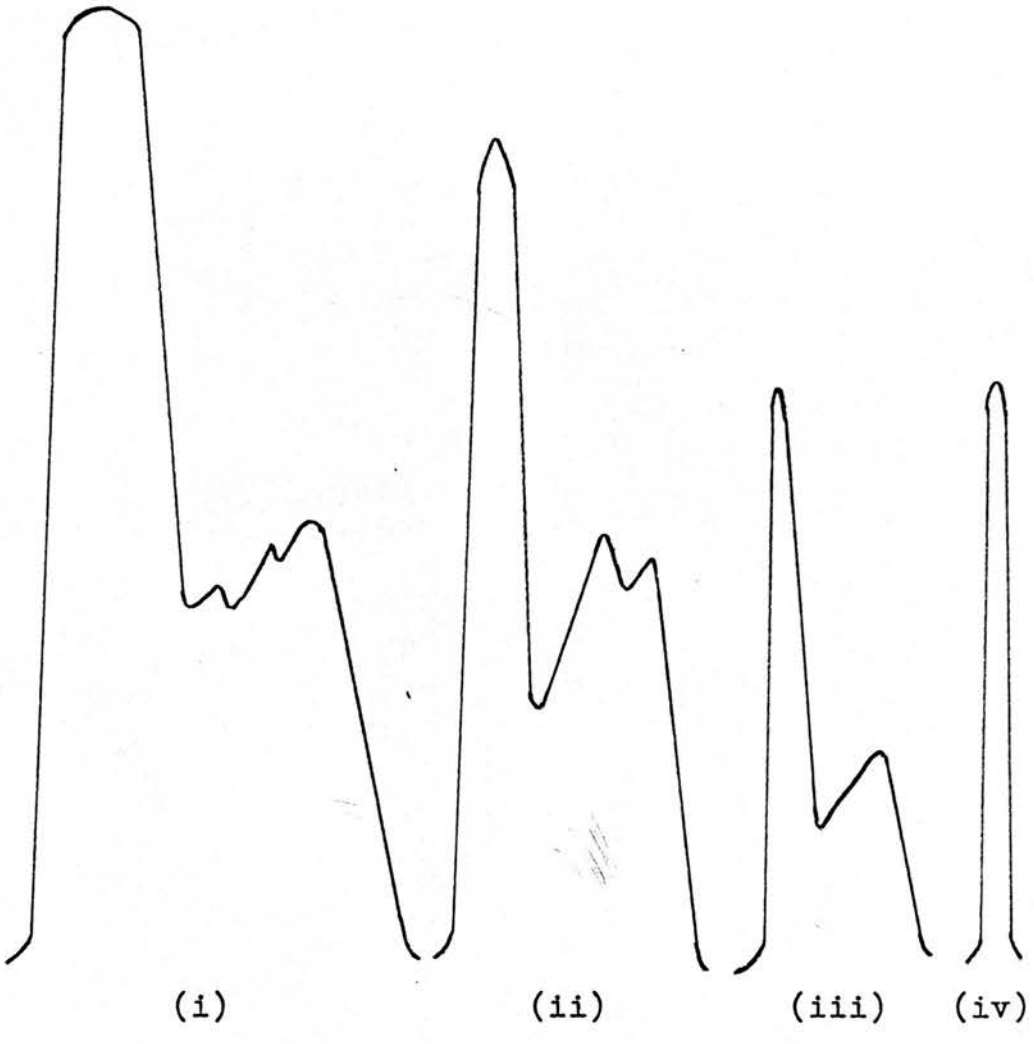
3. Purity of Sephadex G-200 eluate and purification effected by individual treatments

Samples containing equivalent quantities of ferritin iron (3.8µg) were subjected to electrophoresis at pH 8.5 after each stage of the isolation procedure. It can readily be seen (Fig. 2.4) that each treatment removed considerable amounts of contaminating proteins. The final product (Fig. 2.4) ran as a single band which stained for both protein and iron. This was also true when a larger quantity (10µg ferritin iron) was applied to the gel suggesting the absence of minor impurities. Employing the conditions for Sephadex G-200 chromatography described above, all the 10 ferritin fractions were electrophoretically homogeneous. When a shorter column (1 cm^2 x 30 cm) was used only the first 4 of the 8 fractions collected were electrophoretically pure. Electrophoresis at pH 7.9 (Drysdale and Munro 1965) and at pH 9.3 (Linder-Horowitz et al. 1970) has confirmed the homogeneity of the final product.

When used as a criterion of purity it is desirable to perform gel electrophoresis over as wide a pH range as possible. With ferritin the mobility of the protein at pH values lower than those used above is negligible. Under the above conditions contaminating proteins more

FIG. 2.4

Electrophoretic Profile of Ferritin Solutions at Various Stages of Isolation Procedure



(i) After CM-cellulose chromatography

(ii) After heat coagulation

(iii) Before Sephadex G-200 chromatography

(iv) Sephadex G-200 eluate.

Samples contained equal quantities of ferritin iron (3.8μg). Electrophoresis was performed at pH 8.5 and gels were stained with Naphthalene Black and scanned at 580 nm (p.27).

basic than ferritin might remain undetected although such impurities might be reasonably expected to be removed by CM-cellulose chromatography.

4. Iron recovery at each stage

The iron of ferritin is a readily measurable parameter which can be used for partial determination of the overall efficiency of the process. It must, however, be emphasised that evaluation of iron recovery gives no information on the recovery of apoferritin. Immunoprecipitation of the heat supernatant has been employed in the determination of the total ferritin protein content (including native apoferritin) of a tissue homogenate but non specific co-precipitation of contaminating proteins can result in anomalously high estimates (Linder and Munro 1972). Similar errors might well occur at later stages in the isolation as long as there was heavy contamination with other proteins. In the absence of a suitable assay procedure for apoferritin during the isolation, only its presence in the final product can be taken as confirmation that the process does not discriminate between low iron or apoferritin and iron rich ferritin. Evidence for this is presented later (p.39).

Table 2.7 presents some typical recoveries obtained with the new procedure. It was previously shown (Table 2.3) that all the non haem iron measured in the pH 4.8 supernatant was immunoprecipitable and that the extraction

TABLE 2.7

Recovery of Ferritin Iron at Various Stages of the Isolation

Experiment	Overall Yield after CM-cellulose Chromatography (per cent)	Overall Yield after Heat Coagulation (per cent)	Yield from $(NH_4)_2SO_4$ Precipitation (per cent)	Yield from Sephadex G-200 Chromatography (per cent)	Total Overall Yield (per cent)
1	86	83	87	100	62
2	92	89	92	100	75
3	92	88	90	99	72
4	98	88	85	100	73
5	98	98	89	not calculated	87
6	89	84	89	"	66
7	92	90	86	"	71
	mean 92.4 ± 4.7(SD)	mean 88.6 ± 4.9(SD)	mean 88.3 ± 2.4(SD)		mean 72.3 ± 7.9(SD)
8	93	87	94	"	76
9	83	79	99	"	65

In Experiments 1-7 $(NH_4)_2SO_4$ precipitation was performed at a level of 50 per cent saturation and in Experiments 8 and 9 a level of 60 per cent saturation was used.

of this iron from the homogenate was quantitative. The iron determined at each subsequent stage has also been demonstrated to be immunoprecipitable thus providing further evidence that the yields recorded are representative of ferritin iron. The overall efficiency of the process is of the order of 70 per cent. There are few published recoveries available for comparison, but Linder and Munro (1972) have quoted 25 per cent.

5. Characterisation of the final product

The electrophoretic behaviour of the final product appeared characteristic of ferritin in that the slow moving brown band, readily observable in the gel, stained for both protein with naphthalene black and for iron by the Prussian Blue reaction with potassium ferrocyanide. However, additional slower migrating bands which have been considered by other workers to be representative of ferritin oligomers have not been routinely observed in the present work. Precipitation by ferritin antiserum (p. 37) also indicated that the final product exhibited properties typical of the ferritin molecule. The product has been further characterised by (i) measurement of the average number of iron atoms per molecule of protein for males and females and (ii) density gradient centrifugation.

(i) Measurement of the average number of iron atoms per molecule of protein.

A useful indication of the composition of ferritin is obtained by measurement of the average number of iron atoms per molecule of protein. Niitsu and Listowsky (1973) demonstrated that the predominant molecular species for both horse spleen and rat liver ferritins contained 2000 iron atoms per molecule of protein. Linder and Munro (1972) obtained a value of 2990 iron atoms per molecule of protein with very little variation for the adult rat. Bjorklid and Helgeland (1970) have reported values of 1750-2640 and 1890-3640 iron atoms per molecule of protein for males and females respectively. Ferritin isolated by the new procedure has yielded values of 1815 ± 330 (17 animals) and 2400 ± 310 (18 animals) for males and females respectively.

The values obtained in this study are similar to those obtained by Bjorklid and Helgeland (1970). Since these workers estimated ferritin protein by a radial immunodiffusion technique on the heat supernatant (70-74 C, 10 mins) of the crude homogenate which was not subjected to any further treatment, the potential loss of apoferritin and ferritin would be minimal. From this comparison it would, therefore, appear that ferritin purified by the new procedure is representative of the whole molecular population.

(ii) Sucrose density gradient centrifugation

The heterogeneous nature of ferritin in the ultracentrifuge was first demonstrated by Rothen (1944) who

fractionated apoferritin from the denser iron containing ferritin molecules. Apoferritin has since been prepared from native ferritin solutions by density gradient centrifugation (Fischbach and Anderegg 1965, Niitsu and Listowsky 1973). Drysdale and Munro (1966) although unable to obtain the apoferritin precursor, isolated low iron containing ferritin from a sucrose density gradient. In the present study the presence of apoferritin or low iron ferritin in the purified preparation has been sought by application of the Drysdale and Munro (1966) procedure.

Procedure:

A sucrose gradient (0-0.25 mol/ℓ) was prepared using a Buchler Auto Densi-Flow apparatus. The electrophoretically homogeneous ferritin solution (0.4 ml) was carefully layered on top of the gradient (4.6 ml) and centrifugation was performed at 80,000g for 90 minutes. The 3 x 7 ml swing out rotor of the Martin Christ Omikron centrifuge was used. Fractions (0.75 ml) were collected using the Buchler apparatus and each was dialysed against phosphate buffer (0.01 mol/ℓ) pH 7.0 to remove sucrose prior to nitrogen estimation. Iron determinations were also made. The results of two such experiments are depicted in Table 2.8.

In both trials an iron-free, nitrogen containing substance was detected in the fraction of lowest density. The average nitrogen content of this fraction was about

TABLE 2.8

Sucrose Density Gradient Centrifugation of Ferritin

	Fraction Number	Per Cent of Total Nitrogen	Per Cent of Total Fe	Fe:N Ratio	Fe Atoms/ Mole Protein
	1	5.9	None	None	None
	2	8.6	5.2	1.07:1	1345
Experiment	3	33.7	32.6	1.65:1	2074
I	4	18.5	21.4	2.04:1	2565
	5	21.1	25.8	2.14:1	2690
	6	12.2	15.0	2.17:1	2728
	1	2.9	None	None	None
	2	12.2	6.1	1.16:1	1458
Experiment	3	28.0	26.7	2.19:1	2753
II	4	25.7	25.6	2.28:1	2866
	5	20.2	21.2	2.40:1	3017
	6	11.0	20.4	2.42:1	3042

A purified ferritin solution was centrifuged (80,000g, 90 min) on a sucrose gradient (0-0.25 mol/ℓ). Six fractions were collected from the gradient and each was assayed for iron (μg/ml) and nitrogen (μg/ml). The iron to nitrogen ratio (g/g) and the average number of iron atoms per molecule protein were calculated.

4.5 per cent of the total nitrogen measured. In view of the previously determined electrophoretic homogeneity of the ferritin preparation, this nitrogen has been tentatively assigned to apoferritin. Fraction number 2 contained on average about 10.4 per cent of the total nitrogen and 5.7 per cent total iron. The amount of apoferritin recovered is much lower than the 19-26 per cent estimated by Rothen (1944). However, the present value is only approximate inasmuch as the fraction size was arbitarily chosen at 0.75 ml and apoferritin might, therefore, also be present in the second fraction. Niitsu and Listowsky (1973) estimated ferritin containing less than 50 iron atoms per molecule of protein to be of the order of 20 per cent. The much smaller quantities of low iron ferritin and apoferritin found in this work might be partly attributed to the use of female animals. As has been already noted (p.13) the female generally stores more iron in the liver than the male and evidence from this work suggests that female ferritin contains on average more iron atoms per molecule protein. These factors might influence the amount of apoferritin present in the liver but unfortunately a similar experiment has not been performed with males. The present experiment does suggest that apoferritin and low ironferritin has not been discarded during the isolation and that the preparation is consistent with a heterogeneous molecular population.

6. Reproducibility of the procedure

One criterion of the usefulness of any procedure is its reproducibility.

The livers of 3 female rats (average body weight 185g) were excised and cut into slices of approximately 1g weight. A composite mixture of these slices was prepared and the whole of the sample was equally divided into three portions. Ferritin was isolated from each portion and the preparations were analysed separately. Table 2.9 gives the results of two such trials.

Within the limits of experimental error, the procedure appeared satisfactorily reproducible.

7. Recycling of the purified ferritin

Recycling experiments have been performed to assess the reliability of the procedure. Low iron ferritin and probably apoferritin have been shown to be present in the Sephadex G-200 eluate. If the stability of this molecular population was adversely affected during the first isolation, a second similar treatment might be expected to exacerbate the situation. Such an occurrence would be reflected in changes in the number of iron atoms per molecule of protein of the recycled material.

Ferritin was isolated and its iron to nitrogen ratio was measured. This protein solution was subjected to exactly the same treatments for a second time and the iron to nitrogen ratio was again determined. The following

TABLE 2.9

Reproducibility of Procedure - Ferritin Iron Assay and Iron to Nitrogen Ratio

		Ferritin Fe	Fe:N Ratio	Fe Atoms/ Mole Protein
Experiment I	Replicate 1	8.5	1.78:1	2240
	Replicate 2	8.7	1.84:1	2310
	Replicate 3	8.1	1.78:1	2240
Experiment II	Replicate 1	10.4	2.15:1	2700
	Replicate 2	10.3	2.18:1	2740
	Replicate 3	10.2	not calculated	not calculated

3 separate homogenates were prepared from the composite mixture of rat livers and ferritin iron (mg/100g wet tissue) was measured in the pH 4.8 supernatant of each. Ferritin was isolated from each homogenate and the Fe:N ratio (g/g) was determined on the purified protein and the average number of iron atoms per molecule protein was calculated (p. 26).

observations were recorded: i) the recovery of ferritin iron from the CM-cellulose column was 85 per cent; ii) when the CM-cellulose eluate was heated during recycling the final product showed the same iron to nitrogen ratio as when this step was omitted, indicating that heat treatment did not discriminate between different molecular species during recycling; iii) when the heat treated CM-cellulose eluate was precipitated by the addition of solid ammonium sulphate to give a final saturation of 60 per cent no appreciable change in the iron to nitrogen ratio of the Sephadex G-200 eluate from that of the original preparation was observed. Before recycling the ratio was 1.95:1 (2450 Fe atoms/mol protein), after recycling this was 2.11:1 (2650 Fe atoms/mol protein). The latter figure was obtained when the ammonium sulphate precipitate was chromatographed on either Sephadex G-200 (1 cm^2 x 60 cm) or on Sephadex G-25 (1 cm^2 x 30 cm) suggesting that any molecules which may have been denatured by preceding treatments were not preferentially retained on Sephadex G-200 during the 4 hr. chromatography.

In the recycling experiments it has been found that if re-precipitation was accomplished by the addition of an equal volume of saturated ammonium sulphate an increase in the iron to nitrogen ratio of the Sephadex G-200 eluate occurred 2.10→ 2.48:1 (2640→ 3120 Fe atoms/mol protein). This indicates incomplete recovery of apoferritin or low iron ferritin. In the present instance the effect could

possibly be magnified because there are no contaminating proteins present which might have aided co-precipitation of apoferritin or low iron ferritin. Crichton et al. (1975) reported that in mucosal tissue in which the iron content of ferritin is low, ammonium sulphate fractionation resulted in very poor yields. However, no figures for the yields or the percentage ammonium sulphate saturation were given.

The observations from the recycling experiments suggest that, within the limits of experimental error, the conditions of isolation do not involve discrimination against the less stable molecular population to any marked extent.

8. Comparisons with other procedures

The efficiency of the newly developed procedure has been directly compared with that of Drysdale and Munro (1965). In their procedure heat coagulation was performed at 80 C as described and also at a lower temperature (70 C). The overall yields were 87, 55 and 31 per cent for the new procedure, 70 C coagulation and 80 C coagulation respectively. The lower yields could be attributed to incomplete retrieval of ferritin iron in the heat supernatant and losses on lowering to pH 4.8 (p.32) and during CM-cellulose chromatography.

It has been reported (Alpert et al. 1973) that pure ferritin was obtained despite the omission of CM-

cellulose chromatography. Another group also omitted this step (Linder-Horowitz et al. 1970). In view of these reports the necessity for ion exchange chromatography was re-assessed by a comparison between the new procedure and that of Linder-Horowitz et al. (1970), which was followed exactly except that a longer column of Sephadex G-200 (1 cm^2 x 60 cm) was employed. The electrophoretic homogeneity and the iron to nitrogen ratio of the product from each isolation was determined.

When the procedure of Linder-Horowitz et al. (1970) was tested, a turbidity was found in the initial Sephadex G-200 ferritin fractions. On electrophoresis this "milkiness" could be seen to remain on top of the gel and did not migrate into it. This turbidity is not seen when CM-cellulose chromatography is included in the procedure. All the ferritin fractions exhibited a protein band in addition to that of ferritin. The measured iron to nitrogen ratios for the products obtained by the Linder-Horowitz et al. (1970) procedure and the new procedure were 1:1.33 and 1:0.55 respectively confirming the presence of nitrogenous impurity in the former. These observations justified the inclusion and continued use of CM-cellulose and it may be noted that in recent work Drysdale (1974) has re-instated this stage. Linder and Munro (1972) have reported that the procedure of Linder-Horowitz et al. (1970) gives ferritin sufficiently pure for immunoprecipitation analysis.

Suran and Tarver (1965), and Niitsu and Listowsky (1973) have reported the fractionation of ferritin by stepwise elution from ion exchange columns. Fractionation observed by such methods may be artificial, but in any case the high ionic strength of the buffer used in the new procedure was chosen to prevent fractionation of ferritin whether real or artificial.

Penders et al. (1968), Bjorklid and Helgeland (1970) have described the isolation of ferritin by a technique involving differential centrifugation. It was thought that a technique of this nature might have entailed a risk of discriminating between iron-rich and iron-poor ferritins. When ferritin prepared by the present procedure was centrifuged according to the conditions of Bjorklid and Helgeland the iron to nitrogen ratio rose from an initial value of 2.02:1 (2540 Fe atoms/mol protein) to 2.44:1 (3070 Fe atoms/mol protein) after centrifugation. This observation suggested that ultracentrifugation discriminated between iron-rich and iron-poor ferritins and provided indirect evidence that the latter population was present in the CM-cellulose preparation.

CHAPTER 3

DIETS AND DESIGN OF EXPERIMENTS

Diets

Albino random bred Wistar rats of both sexes were used in the experiments on the effects of prolonged protein deficiency upon liver ferritin metabolism. They were fed synthetic diets which were initially formulated as described by Bethard et al. (1958) and Achmed (1965) and designed to supply a daily intake of 3-4 mg iron per rat. The control and protein deficient diets were identical except that the former contained 22 per cent casein while in the latter this was replaced by an equicaloric quantity of carbohydrate. The original workers used dextrin as the sole source of carbohydrate but the only dextrin now available gave diets which became sticky and indeed unmanageable by the rats in the presence of water. The animals fed the protein deficient diet found it extremely unpalatable and quickly showed signs of severe inanition within a few days. Young males fed the complete diet also ate barely sufficient to maintain their body weight. These difficulties were overcome to some extent by omitting water from the formulation and by replacing half of the dextrin in the diet by starch and adding 6 per cent of sucrose without changing the overall carbohydrate content. The problem of palatability however, could only be solved by including yeast extract (2.3 per cent) to the diet. The contribution of this to the supply of protein (approximately 0.7 per cent) and vitamins (approximately 12 mg niacin per 1,000g diet, 1.5 mg ribo-

flavin per 1,000g diet) was ignored. The vitamins were added individually to the diet with the exception of vitamins A and D which were supplied in a commercial preparation as an addition to the drinking water. The formulation of the diets and the compositions of the salt mix and vitamins are given in Tables 3.1, 3.2, 3.3.

The synthetic diets were given in powder form in open dishes which were replenished daily and the approximate daily food consumption was recorded. The animals were allowed free access to drinking water.

Evaluation of Diets

The two main requirements of the control diet are the ability to support growth and the ability to give normal iron storage, whereas the performance of the protein deficient diet can be assessed by its failure to sustain growth despite an adequate intake of energy and other recognised growth factors. Since low values for serum transferrin have been reported for protein deficient rats (Achmed and Ramsay 1974) and in human kwashiorkor (Lahey et al. 1958, Adams and Scragg 1965, El-Shobaki et al. 1972, MacFarlane 1969) some measurements of this have been included.

Since it seemed desirable to establish the quality of the modified control diet at the outset, its capacity to promote growth of young male rats (60g) over a five

TABLE 3.1

Composition of Synthetic Diets

	Casein Diet (g)	Protein Deficient Diet (g)
Dextrin	21.9	32.9
Starch	21.9	32.9
Sugar (Sucrose)	5.0	5.0
Casein	22.0	Nil
Corn Oil	4.0	4.0
Agar	5.0	5.0
Salt Mixture	4.0 (Table 3.2)	4.0 (Table 3.2)
Yeast Extract*	2.0	2.0
Vitamins	(Table 3.3)	(Table 3.3)

* Soluble yeast extract (Oxoid)

TABLE 3.2

Composition of Salt Mixture

U.S.P. (1955) XV 883

	(g)
Sodium Chloride ($NaCl$)	139.30
Potassium Dihydrogen Phosphate (KH_2PO_4)	389.00
Magnesium Sulphate ($MgSO_4.7H_2O$)	57.30
Calcium Carbonate ($CaCO_3$)	381.00
Ferrous Sulphate ($FeSO_4.7H_2O$)	27.00
Manganese Sulphate ($MnSO_4.4H_2O$)	4.00
Zinc Sulphate ($ZnSO_4.7H_2O$)	0.55
Potassium Iodide (KI)	0.97
Cupric Sulphate ($CuSO_4.5H_2O$)	0.48
Cobaltous Chloride ($CoCl_2.6H_2O$)	0.02

TABLE 3.3

Vitamins

α-Tocopherol	100
Menadione	50
Thiamine	22
Riboflavin	22
Pyridoxine	22
Niacin	100
Calcium Pantothenate	66
Choline Chloride	1,650
Inositol	100
p-Amino Benzoic Acid	100
Ascorbic Acid	500

The Vitamins (mg per 858g diet) were incorporated directly into the diet.

Vitamins A and D were supplied in commercial product "Adexolin" (Boots Company Limited, Nottingham, England) which was administered in the drinking water. 3 drops per 250 ml were given and the rats consumed on average 175 IU Vitamin A/day/rat, 17.5 IU Vitamin D/day/rat.

week period was compared with that of the laboratory stock diet. The animals fed the experimental casein diet gained weight at an average rate of 15g/week, whereas the average weight gain for those fed the stock diet was 18g/week. Although the stock rats grew somewhat more quickly than the experimental animals it was considered that the composition of the control diet was at least good enough to justify further study.

Further evidence on the suitability of the diets was obtained in the course of the main experiments which were performed in the following way.

Design of Experiments

Five animals were studied in each group. This number, albeit small from a statistical viewpoint was the maximum that one operator could satisfactorily deal with. Five experiments have been performed, two with males and three with females. Normally the animals were maintained on the diets for five weeks but one trial with females was extended to six weeks. No precautions against coprophagy were taken. The animals were weighed weekly and at the end of the experimental period were killed under ether anaesthesia. The livers were quickly removed, blotted dry with a paper tissue, weighed and frozen (-10 C) until use. Normally they were used the same day but always within a period of three days and were analysed

for total non haem iron and ferritin iron (mg/liver). Pure ferritin was isolated from the livers and used for iron and nitrogen determinations from which the number of iron atoms per ferritin molecule was calculated (p.26). Ferritin protein (mg/liver) was calculated as described earlier (p.26) from the iron to nitrogen ratio of the purified protein and the ferritin iron content of the liver homogenate. Five animals were killed at the outset of each experiment and were similarly treated.

In Experiment I male rats weighing 375-435g (age 120-130 days) were housed together and fed the diets ad libitum. In Experiment II males weighing 285-345g (age 70-80 days) were used. In this instance those receiving the protein deficient diet were housed individually and an additional group was included which was pair-fed the casein diet. The animals in the latter group were housed individually and were matched with those of an initially similar body weight which received the protein deficient diet. The food intake of this control group was then limited to the amount eaten by their protein deficient counterparts.

Experiment III was continued for six weeks using females weighing 190-210g (age 70-80 days). In Experiment IV heavier females (210-260g, age 120-130 days) were used; in both Experiments III and IV the animals were housed together and fed the diets ad libitum. In Experiment V females (195-235g) on both the protein

deficient and control diets were housed individually and the food intake of the latter was restricted to the amount eaten by the protein deficient ones.

In all five experiments a group receiving laboratory stock diet was studied as an additional control.

The ability of the casein diet to support growth or maintain the weight of the older rats of both sexes is illustrated in Table 3.4 which also shows the figures for similar animals fed the laboratory stock diet. The same table shows the striking loss in weight for those fed the casein free diet. Table 3.5 gives corresponding values for liver weights. The results of serum transferrin measurements made in Experiments II and V are given in Table 3.6.

These results confirm the earlier observation (p.48) that males fed the laboratory stock diet gain more weight than those fed the experimental casein diet. In Experiment I the smallweight gain of both protein adequate groups most probably reflects a declining rate of growth of older animals. With females the growth rate of the experimental control group and the group fed the laboratory stock diet, although small, was similar. In all five experiments the considerable loss in body weight of protein deficient rats could be attributed mainly to the lack of protein since the daily calorie intake was not greatly diminished from that of normal animals. In agreement with the observation of another group of workers

TABLE 3.4

Growth of Rats on Experimental Diets

Experiment	Diet	Food Intake	Body Weight Initial	Body Weight Final	Overall Weight Change
I Male	- Casein	30	396 ± 17	299 ± 19	-97 (± 2)
	Casein	35	422 ± 13	424 ± 19	+ 2 (± 6)
	Stock	*	419 ± 15	441 ± 26	+22 (±11)
II Male	- Casein	20	292 ± 6	242 ± 13	-50 (± 7)
	Casein (Pairfed)	20	292 ± 6	299 ± 17	+ 7 (±11)
	Casein (ad lib)	30	291 ± 5	340 ± 23	+49 (±18)
	Stock	*	322 ± 20	401 ± 23	+79 (± 3)
III Female	- Casein	18	202 ± 3	162 ± 7	-40 (± 4)
	Casein	21	195 ± 6	213 ± 13	+18 (± 7)
	Stock	*	201 ± 5	238 ± 18	+37 (±13)
IV Female	- Casein	18	236 ± 19	175 ± 20	-61 (± 1)
	Casein	21	220 ± 6	226 ± 10	+ 6 (± 4)
	Stock	*	231 ± 25	239 ± 26	+ 8 (± 1)
V Female	- Casein	20	211 ± 9	167 ± 7	-44 (± 2)
	Casein (Pairfed)	20	208 ± 10	221 ± 15	+13 (± 5)
	Stock (Pairfed)	20	224 ± 7	236 ± 15	+12 (± 8)

*Not determined

Each group of rats consisted of 5 animals which were kept on the experimental diets for 5 weeks except in Experiment III when this period was 6 weeks. All weights are given in grams ± SD. The average food intake is given in g/rat/day.

TABLE 3.5

Liver Weights (at slaughter) of Rats on Experimental Control and Protein Deficient Diets

Experiment	Diet	Liver Weight	Liver Weight % of Body Weight
I Male	- Casein	8.8 ± 0.5	2.9
	Casein	13.6 ± 1.2	3.2
	Stock	14.5 ± 1.9	3.3
II Male	- Casein	6.6 ± 0.5	2.7
	Casein (Pairfed)	9.6 ± 0.6	3.2
	Casein (ad lib)	10.8 ± 1.0	3.2
	Stock	14.5 ± 1.0	3.6
III Female	- Casein	5.2 ± 0.5	3.2
	Casein	6.5 ± 0.4	3.1
	Stock	8.0 ± 0.6	3.4
IV Female	- Casein	5.8 ± 0.9	3.3
	Casein	6.7 ± 0.5	3.0
	Stock	8.8 ± 1.6	3.7
V Female	- Casein	5.2 ± 0.2	3.1
	Casein (Pairfed)	8.4 ± 0.7	3.8
	Stock (Pairfed)	8.5 ± 0.6	3.6

Each group of rats consisted of 5 animals which were fed the diets for 5 weeks except Experiment III which lasted 6 weeks. The weight of the liver at slaughter is given in grams ± SD.

TABLE 3.6

Serum Transferrin in Protein Deficient and Experimental Control Rats

Experiment	Diet	Transferrin
II Male	Protein Deficient	28.6 ± 2.5
	Casein Pair fed	44.9 ± 2.9
	Casein ad lib	44.9 ± 2.5
	Stock	48.5 ± 2.8
V Female	Protein Deficient	30.9 ± 2.9
	Casein Pair fed	46.4 ± 4.3
	Stock	51.2 ± 4.3

Serum transferrin (μmol/ℓ ± SD) was determined from the assay of the total iron binding capacity (Ramsay 1975). Since transferrin binds 2 moles iron per mole protein the iron binding capacity figures were divided by a factor of 2. Five animals per group were studied. Each animal under ether anaesthesia was bled from the heart.

(Achmed and Ramsay 1974) the serum transferrin levels of the rats fed the casein free diet were much below those of both the protein replete groups.

The extent to which the casein diet gives normal iron storage compared with the laboratory stock diet is given in Table 3.7. Since much of the liver weight consists of water and cytoplasmic elements not directly involved in iron storage comparisons between groups having different liver weights might be considered less liable to misinterpretation when the values are expressed in absolute amounts. For this reason the results in Table 3.7 are expressed as the quantity of iron present per organ.

The results show that in all the experiments with the females the animals fed the synthetic diet and the laboratory stock diet continued to deposit extra iron in the liver to approximately similar levels. In Experiment II the younger males (70-80 days) resembled females in continuing to deposit iron while on the normal diet, but the older animals (120-130 days) used in Experiment I did not. However, in both these experiments the quantity of iron stored by the animals on the casein diet and laboratory stock diet was approximately similar indicating that the change to the synthetic diet was not the cause of increased storage iron in the younger males.

The ability of the casein diet to support a normal level of ferritin protein compared with that of the

TABLE 3.7

Total Non Haem Iron (TNHI) and Ferritin Iron Content in Livers of Rats on Control Experimental Diets

Experiment	Diet	TNHI	Ferritin Fe	Ferritin Fe % of TNHI
I Male	Initial Stock*	1.24 ± 0.21	0.97 ± 0.20	78.5 ± 5.1
	Casein	1.61 ± 0.34	1.19 ± 0.23	74.8 ± 2.7
	Stock	1.31 ± 0.14	0.91 ± 0.09	69.5 ± 3.2
II Male	Initial Stock*	0.76 ± 0.12	0.55 ± 0.09	73.0 ± 5.7
	Casein (Pairfed)	1.40 ± 0.10	1.06 ± 0.10	75.9 ± 1.7
	Casein (ad lib)	1.39 ± 0.17	1.17 ± 0.13	83.5 ± 5.1
	Stock	1.17 ± 0.31	0.92 ± 0.25	79.4 ± 1.9
III Female	Initial Stock*	1.01 ± 0.20	0.80 ± 0.21	79.4 ± 3.5
	Casein	2.06 ± 0.35	1.76 ± 0.44	85.1 ± 2.8
	Stock	2.22 ± 0.43	1.85 ± 0.35	83.4 ± 3.2
IV Female	Initial Stock*	1.27 ± 0.11	1.10 ± 0.09	86.9 ± 4.0
	Casein	2.15 ± 0.63	1.69 ± 0.42	79.1 ± 7.8
	Stock	2.35 ± 0.56	2.13 ± 0.54	90.4 ± 4.0
V Female	Initial Stock*	1.66 ± 0.42	1.39 ± 0.31	85.5 ± 3.8
	Casein (Pairfed)	2.70 ± 0.65	2.06 ± 0.42	76.8 ± 4.6
	Stock (Pairfed)	2.29 ± 0.52	1.76 ± 0.33	77.8 ± 3.0

*Figures were obtained from animals killed at the outset.

Each group of rats consisted of 5 animals which were fed the diets for 5 weeks except in Experiment III which lasted 6 weeks.

Iron contents are expressed as mg/liver ± SD.

stock diet has also been tested. The quantities of ferritin protein per liver for both groups is given in Table 3.8. In all the experiments the experimental diet is seen to be at least as effective as the laboratory stock diet.

In conclusion, in the main parameters which have been tested the experimental casein diet has proved an adequate substitute as a control protein replete diet for experiments on protein deficiency. The protein deficient diet has also been shown to be adequate, and since the animals fed this diet consumed only slightly less than controls fed ad libitum, it suggests that a calorie deficiency does not compound the adverse effect of protein deprivation. Finally, with respect to iron storage, the continued deposition of extra iron in the livers of female rats maintained on both the protein replete diets does not appear unusual since it is well established that females deposit iron in the liver at a greater rate and to a greater extent than males (Widdowson and McCance 1948, Kaldor and Powell 1957, Bjorklid and Helgeland 1970). A similar tendency in the younger males might be attributed to the particular strain of rat used since as was mentioned previously Bjorklid and Helgeland (1970) have reported that the male rat continued to deposit iron in the liver up to at least 16 weeks of age.

TABLE 3.8

Liver Ferritin Protein Content of Rats on Control Experimental Diets

Experiment	Diet	Ferritin Protein
I Male	Initial Stock*	3.55 ± 0.54
	Casein	4.54 ± 0.54
	Stock	3.04 ± 0.48
II Male	Initial Stock*	1.95 ± 0.44
	Casein (Pairfed)	4.68 ± 0.16
	Casein (ad lib)	6.27 ± 1.29
	Stock	4.86 ± 0.47
III Female	Initial Stock*	2.44 ± 0.23
	Casein	5.95 ± 1.35
	Stock	5.75 ± 1.30
IV Female	Initial Stock*	3.37 ± 0.56
	Casein	5.30 ± 1.06
	Stock	6.37 ± 1.91
V Female	Initial Stock*	4.43 ± 0.64
	Casein (Pairfed)	7.07 ± 0.94
	Stock (Pairfed)	5.81 ± 0.94

* Figures obtained from animals killed at the outset.

Each group of rats consisted of 5 animals which were fed the diets for 5 weeks except in Experiment III which lasted 6 weeks. Ferritin Protein content is expressed in mg/liver ± SD.

CHAPTER 4

RESULTS

This chapter records the results of the experiments in which liver ferritin metabolism during a period of prolonged protein deprivation was studied. The results of experiments which confirm earlier work on iron storage (Hallgren 1953, Achmed and Ramsay 1974) and which have extended it to include a comparison of male and female rats and the effects of pair feeding are presented. The remarkable behaviour of both male and female protein deficient rats to accumulate ferritin protein to a level at least equal to that of control animals is shown. A difference between the sexes with regard to the number of iron atoms stored per molecule protein is also shown. Finally a characteristic relationship between iron and protein has been derived which suggests a sex difference in ferritin iron storage between normally fed animals, and the tendency of protein deficient males to (more closely) resemble females.

Liver Ferritin Iron During Protein Deficiency

The values for the control animals which appear in subsequent figures have been presented numerically in the previous chapter (Table 3.7). The results there are expressed as total amounts per whole organ. This form of presentation is continued in this section and in the

section concerning ferritin protein during protein deficiency. The basis for expressing the results in this manner, especially with regard to studies on protein deficiency, has been discussed by Munro and Fleck (1969) and Achmed and Ramsay (1974).

1. Male Rats

Storage Iron:

The effect of prolonged protein deficiency upon ferritin iron storage is shown in Fig. 4.1 in which animals fed the casein control diet are compared with those given the equicaloric protein deficient one.

In Experiment I with older males (120-130 days), the protein deficient animals stored significantly more ferritin iron (1.81 mg SD $\pm$ 0.29) than the casein controls (1.19 mg SD $\pm$ 0.23), ($0.01 < P < 0.02$, $t = 3.351$).

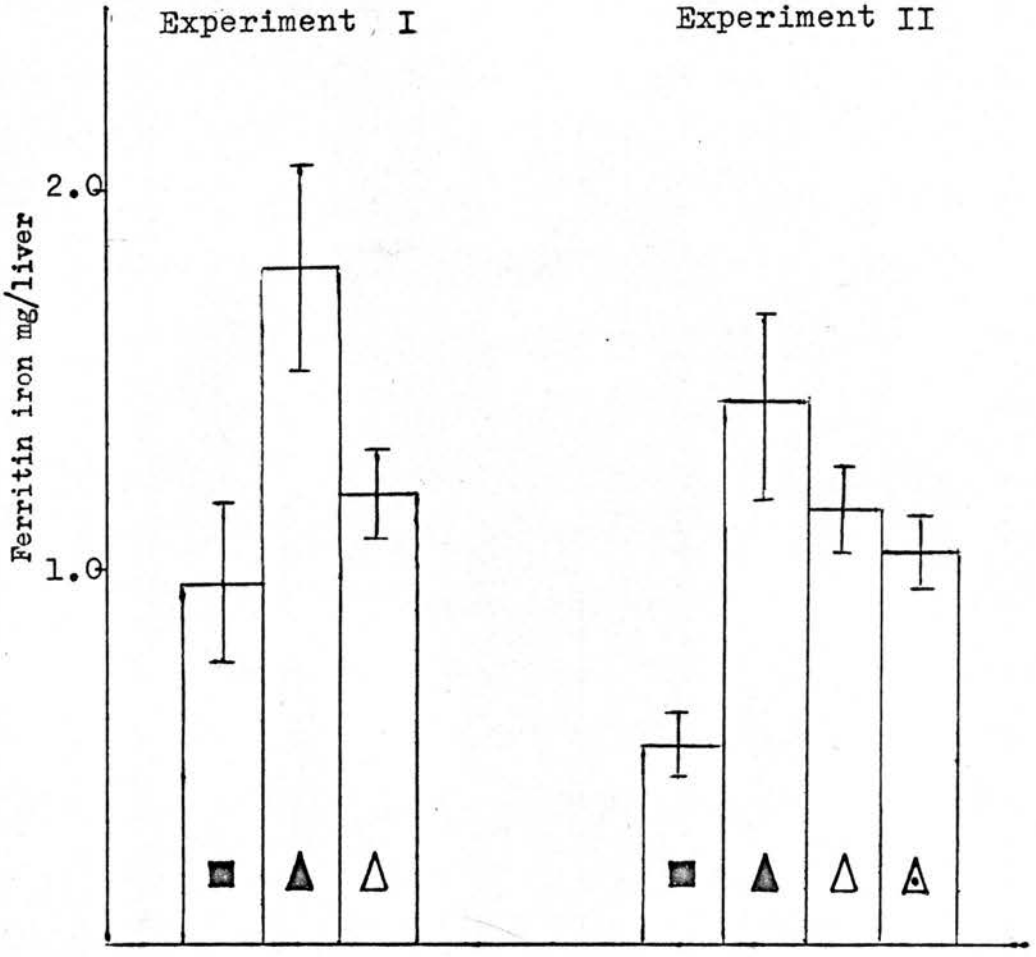
In Experiment II the protein deficient group (1.47 mg SD $\pm$ 0.25) stored more ferritin iron than the pair-fed control animals (1.06 mg SD $\pm$ 0.10), ($P < 0.02$, $t = 2.950$). The difference between protein deficient animals and those given the control diet ad libitum (1.17 mg SD $\pm$ 0.13), ($P < 0.05$, $t = 2.34$) is less convincing probably because they were younger and the controls considerably increased their liver ferritin iron during the experimental period, and also because of the statistical doubt arising from the small number of animals studied.

In both experiments the protein deficient animals stored much more iron as ferritin than those killed at the

FIG. 4.1

Ferritin Iron

Male Rats



■- animals killed at outset ▲- protein deficient
△- casein control, ad lib ⊙- casein control, pair fed

Each group consisted of 5 animals which were fed the experimental diets for 5 weeks.

Iron estimations are given in mg/liver ± SD.

outset. In Experiment I the latter animals stored 0.97 mg SD ± 0.20, and in Experiment II 0.55 mg SD ± 0.09. In the two experiments there was an obvious difference in the iron storage of the control animals. In Experiment I the older animals maintained an almost constant level of ferritin iron with an outset value of 0.97 mg SD ± 0.20 and a value of 1.19 mg SD ± 0.23 at the end of the experiment. In Experiment II the younger males raised the outset level of 0.55 mg SD ± 0.09 to 1.06 mg SD ± 0.10 (pair fed) and 1.17 mg SD ± 0.13 (ad libitum) after 5 weeks.

Percentage non haem iron present as ferritin:

The percentage of non haem iron present as ferritin for the protein deficient and casein control animals is shown in Fig. 4.2.

In Experiment I the level for the protein deficient animals (84.7% SD ± 4.8) was significantly increased over that of the controls (74.8% SD ± 2.7), ($P < 0.01$, $t = 3.596$). In Experiment II, the protein deficient group (86.9% SD ± 3.7) increased the level significantly over those pair-fed the control diet (75.9% SD ± 1.7) but not over the controls fed ad libitum (83.5% SD ± 5.1), ($P > 0.05$, $t = 0.182$).

2. Female Rats

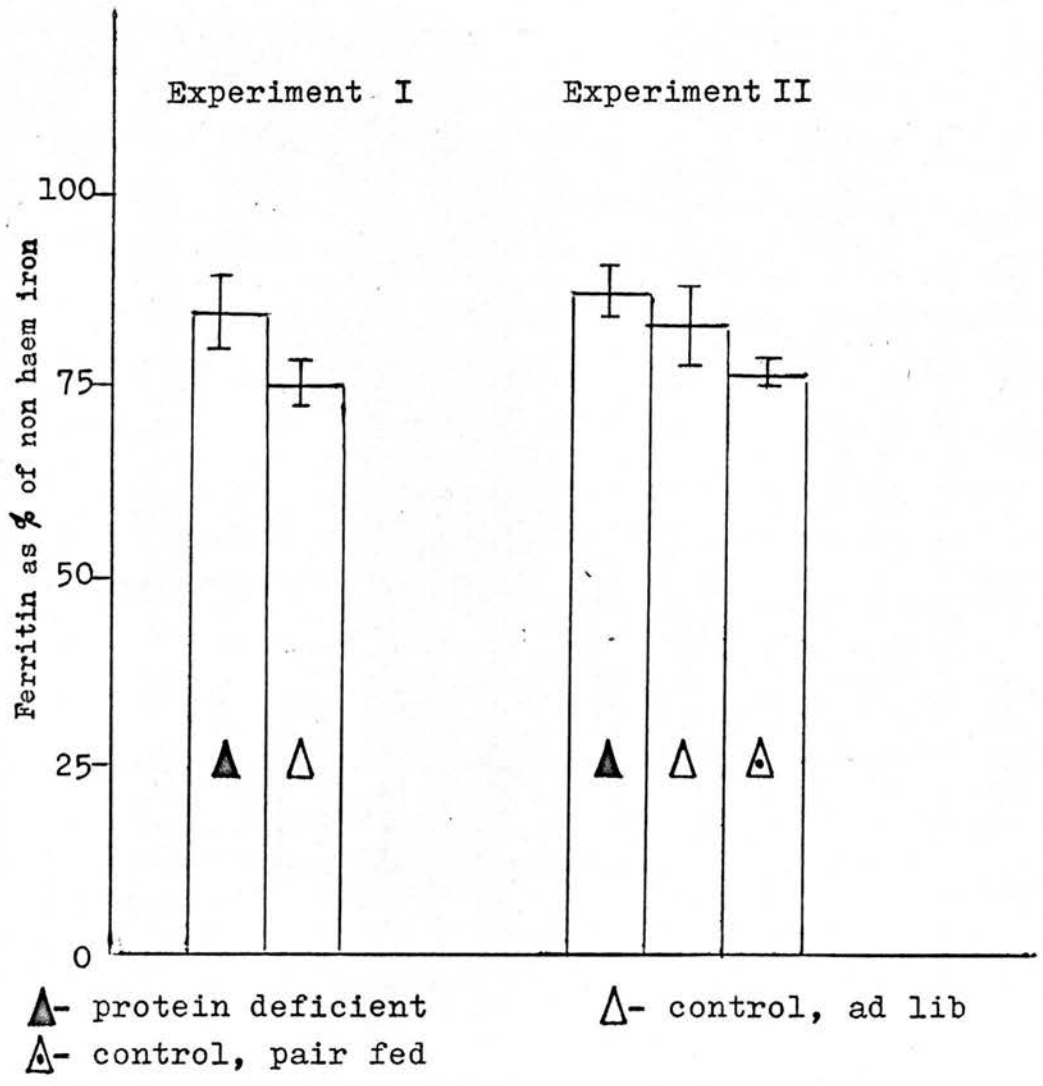
Storage Iron:

The effect of prolonged protein deficiency upon iron stored as ferritin in female rat liver is shown in Fig. 4.3

FIG. 4.2

Ferritin as Percentage of Liver Non Haem Iron

Male Rats



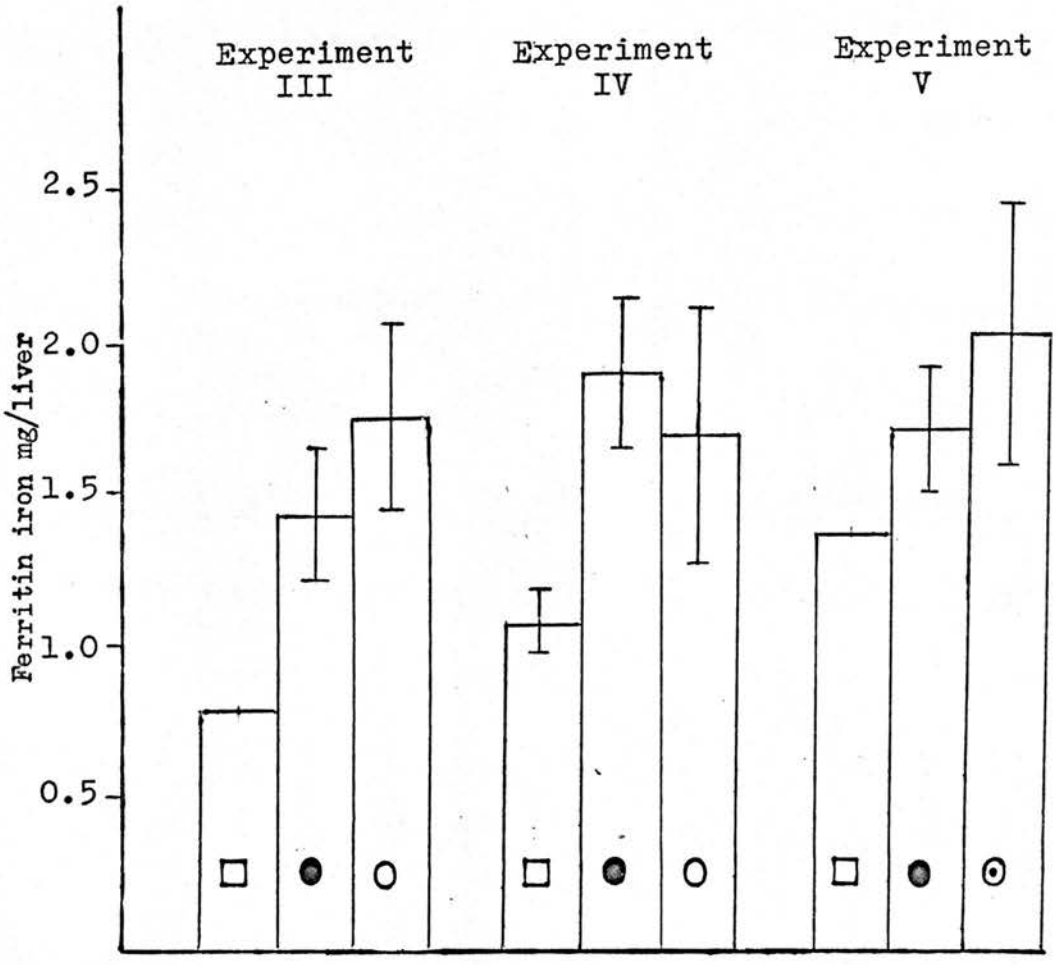
Each group consisted of 5 animals which were pair fed the experimental diets for 5 weeks.

Ferritin is given as a percentage of the total (haemosiderin + ferritin) non haem storage iron.

FIG. 4.3

Ferritin Iron

Female Rats



□- animals killed at outset ●- protein deficient
○- casein control, ad lib ⊙- casein control, pair fed

Each group consisted of 5 animals which were fed the experimental diets for 5 weeks except Experiment III which lasted 6 weeks.

Ferritin iron is given in mg/liver ± SD.

in which animals fed the casein control diet are compared with those receiving the equicaloric protein deficient one.

In Experiment III there was no significant difference ($P > 0.05$, $t = 1.286$) between the protein deficient group (1.44 mg SD $\pm$ 0.23) and the controls (1.76 mg SD $\pm$ 0.44).

In Experiment IV as in the previous experiment there was no significant difference ($P > 0.05$, $t = 0.992$) between the protein deficient group (1.93 mg SD $\pm$ 0.25) and the controls (1.69 mg SD $\pm$ 0.42).

In Experiment V the control animals were pair-fed the control diet. As in the previous two experiments no significant difference was observed between protein deficient (1.73 mg SD $\pm$ 0.19) and control animals (2.06 mg SD $\pm$ 0.42), ($P > 0.05$, $t = 1.425$).

Fig. 4.3 also shows the amount of ferritin iron stored by the animals killed at the outset of the experiment. In Experiments III and IV the animals on the experimental diets stored considerably more iron as ferritin at the end of the experiment but the increased storage during protein deficiency in Experiment V was not quite as convincing.

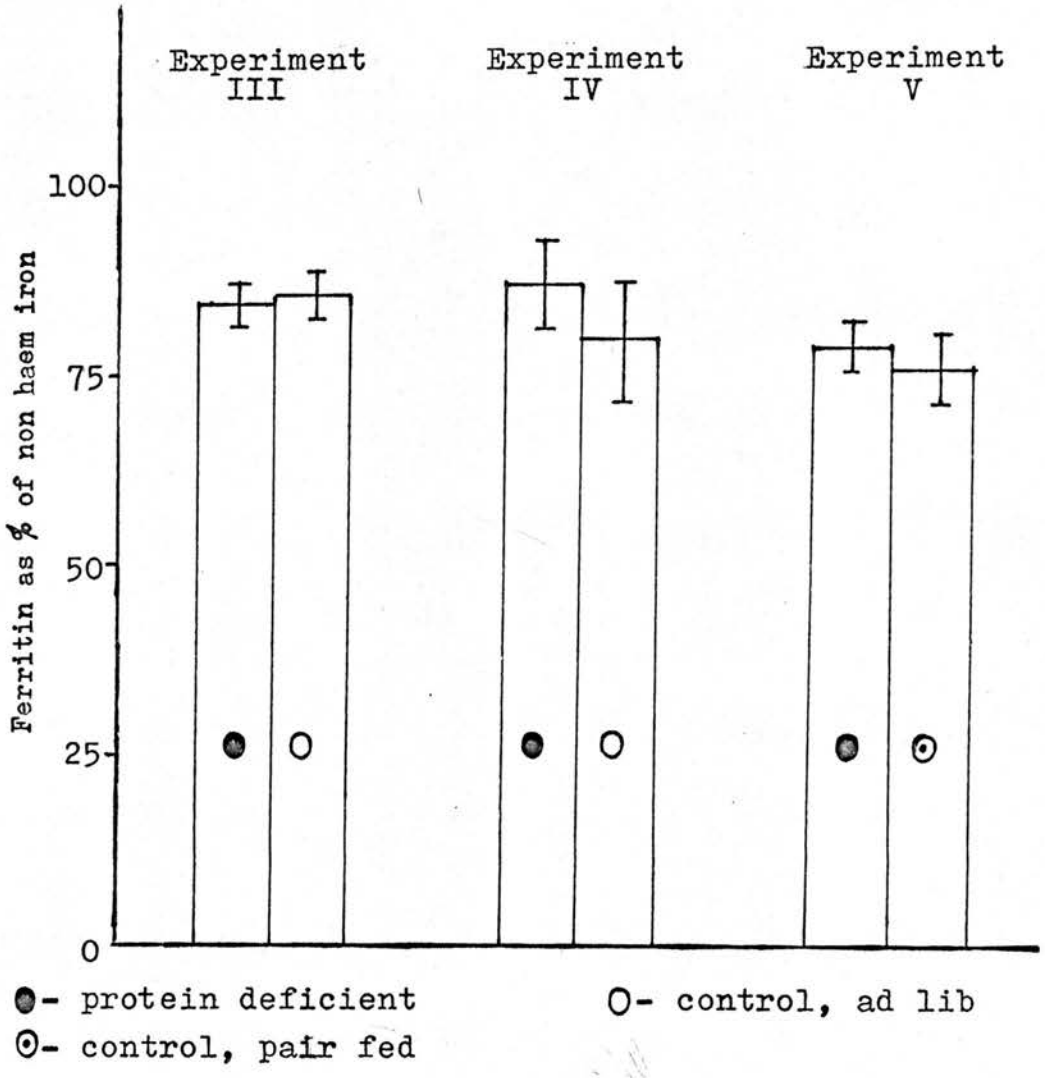
Percentage non haem iron as ferritin:

The percentage non haem iron stored as ferritin is shown in Fig. 4.4. In each of the three experiments there was no significant difference between the protein deficient and casein control animals. In Experiment III the values were 84.0% SD $\pm$ 3.0 (protein deficient), 85.1% SD $\pm$

FIG. 4.4

Ferritin Iron as Percentage of Liver Non Haem Iron

Female Rats



Each group consisted of 5 animals which were fed the experimental diets for 5 weeks except Experiment III which lasted 6 weeks.

Ferritin is given as a percentage of total (haemosiderin+ ferritin) non haem storage iron.

2.8 (casein) $P > 0.05$, $t = 0.394$; in Experiment IV, 86.4% SD ± 6.3 (protein deficient), 79.1% SD ± 7.8 (casein), $P > 0.05$, $t = 1.456$; and in Experiment V, 79.4% SD ± 2.9 (protein deficient), 76.8% SD ± 4.6 (casein), $P > 0.05$, $t = 0.956$.

From these results it is clear that the male rat differs from the female with regard to iron storage. The protein deficient male stores ferritin iron in significantly greater quantities than control animals whereas both the normal and protein deficient females store ferritin iron to the same extent.

Liver Ferritin Protein During Protein Deficiency

The iron to nitrogen ratio (g/g) of purified ferritin isolated from protein deficient male and female rats has been determined and used in the calculation of both the total liver ferritin protein content (p.26) and the number of iron atoms stored per molecule of protein (p.26). Results have been compared with those obtained from suitable control animals whose values have been previously presented (Table 3.8).

1. Male Rats

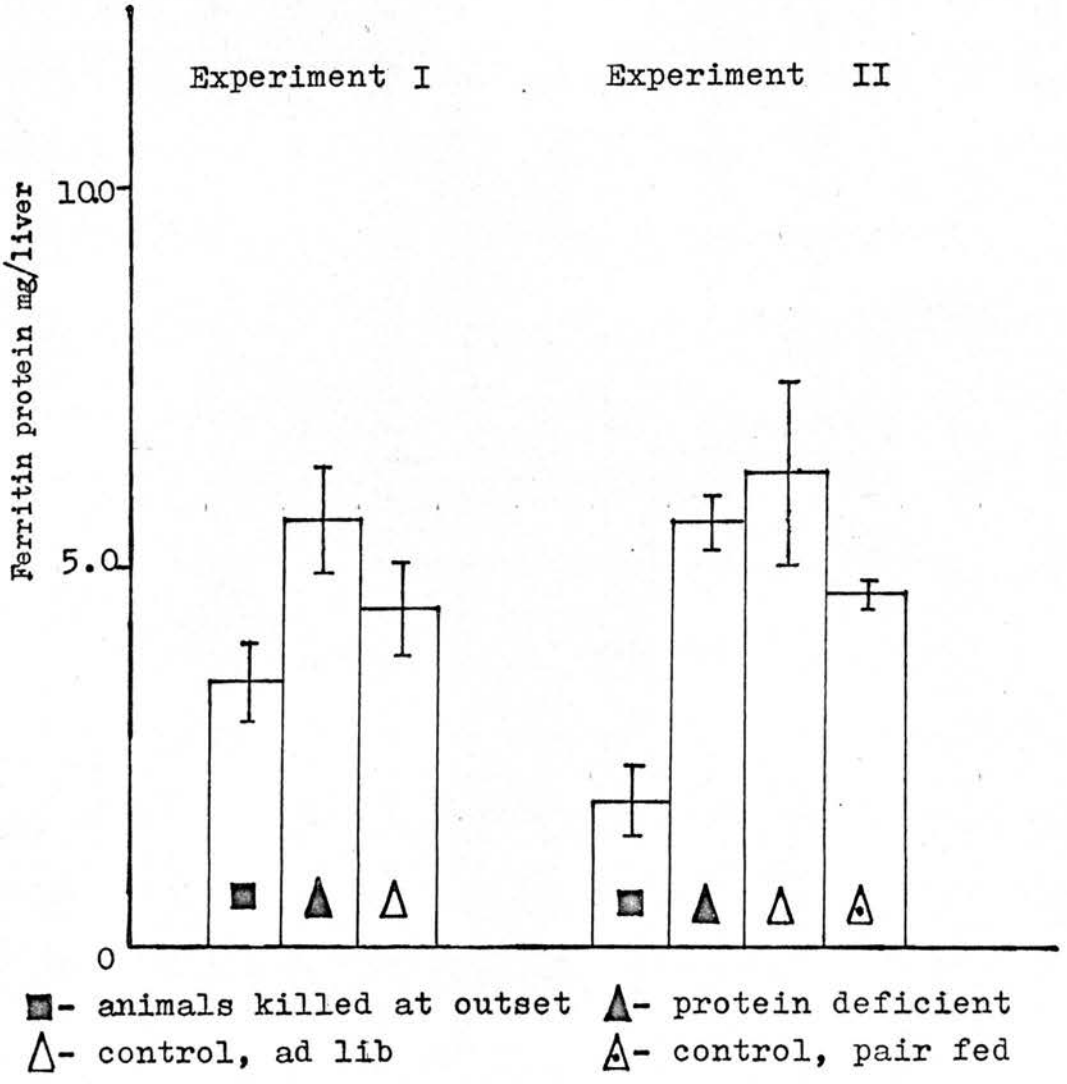
The liver ferritin protein content is shown in Fig.4.5 for the protein deficient and control males for both experiments.

In Experiment I the protein deficient group accumulated 5.67 mg SD ± 0.66 ferritin protein compared with

FIG. 4.5

Ferritin Protein

Male Rats



Each group consisted of 5 animals which were fed the experimental diets for 5 weeks.

Ferritin protein is given in mg/liver ± SD.

4.54 mg SD ± 0.54 for the control group. The difference between the two groups is slightly significant ($0.02 < P < 0.05$, $t = 2.665$). In Experiment II there was no significant difference between the protein deficient group (5.66 mg SD ± 0.33) and the controls fed ad libitum (6.27 mg SD ± 1.29) ($P > 0.05$, $t = 0.917$). However, in the same experiment the protein deficient animals stored significantly more than the pair-fed controls (4.69 mg SD ± 0.16) ($P < 0.01$, $t = 4.90$) as also did the controls fed ad libitum ($0.02 < P < 0.05$, $t = 2.446$).

Thus in both experiments the protein deficient animals were able to maintain their ferritin protein to much the same amount as those on the protein replete control diet and both groups accumulated significantly more ferritin protein during the five week experiment as is shown in Fig. 4.5.

2. Female Rats

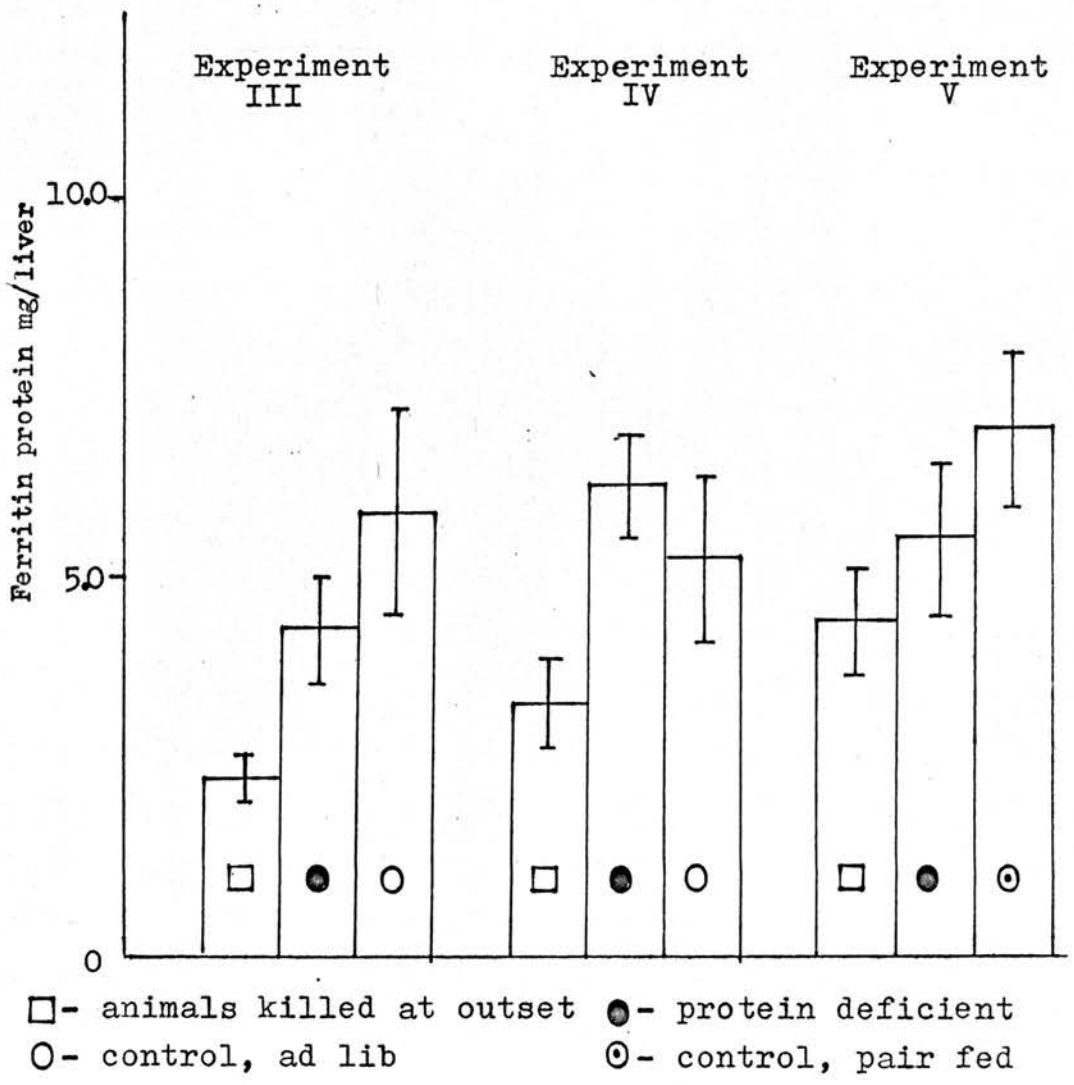
The liver ferritin protein content for the protein deficient and control groups in all three experiments performed with females is shown in Fig. 4.6.

In Experiment III the protein deficient group stored 4.46 mg SD ± 0.68 compared with 5.95 mg SD ± 1.35 for the controls ($P > 0.05$, $t = 2.141$). In Experiment IV the protein deficient animals stored 6.27 mg SD ± 0.62 and the controls 5.30 mg SD ± 1.06 ($P > 0.05$, $t = 1.580$). Pair-feeding the control diet in Experiment V did not alter the general trend in which there was no significant difference

FIG. 4.6

Ferritin Protein

Female Rats



Each group consisted of 5 animals which were fed the experimental diets for 5 weeks, except Experiment III which lasted 6 weeks.

Ferritin protein is given in mg/liver ± SD.

between protein deficient (5.55 mg SD ± 1.06) and controls (7.07 mg SD ± 0.94) ($P > 0.05$, $t = 1.975$).

These results demonstrate that in all three experiments the protein deficient animals have accumulated an amount of ferritin protein that is not significantly different from that of protein replete animals. The results of Fig. 4.6 also show that both groups have increased the levels above that recorded at the outset.

From the above study it can also be concluded that allowing the animals free access to the control diet or restricting them to an amount equal to that consumed by the protein deficient ones had little influence on the overall result, indicating that the lower food intake of the latter was not a major factor in the results.

Relationship Between Iron and Protein in Rat Liver Ferritin

1. Number of iron atoms per ferritin molecule

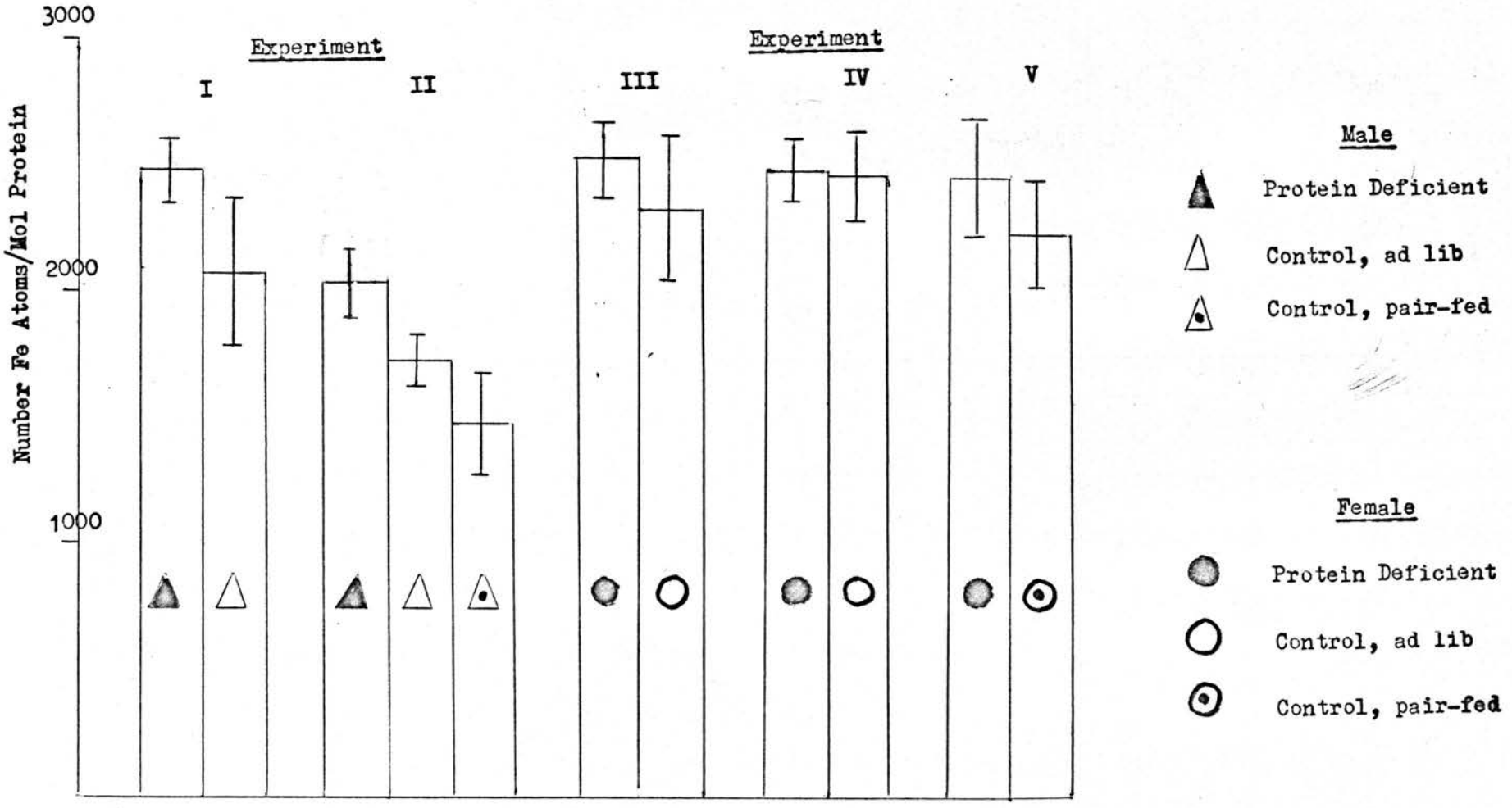
Male Rats:

The number of iron atoms per molecule of ferritin is shown in Fig. 4.7 for the protein deficient and casein control animals in both experiments performed with males.

In Experiment I with older animals (120-130 days) the protein deficient group incorporated significantly more iron into ferritin (2,509 SD ± 136) than the casein controls (2,067 SD ± 300) ($0.02 < P < 0.05$, $t = 2.684$).

FIG. 4.7

Number of Iron Atoms per Molecule Ferritin Protein



In Experiment II the younger males (70-80 days) made protein deficient also incorporated significantly more iron into ferritin (2,026 SD ± 139) than either the pair-fed casein controls (1,738 SD ± 108) ($P < 0.02$, $t = 3.183$) or the controls fed ad libitum (1,489 SD ± 209) ($P < 0.01$, $t = 4.279$). In neither experiment, however, did the increased iron content of the protein approach the theoretical maximum 4,300 iron atoms per molecule (Fischbach and Anderegg 1965).

Female Rats:

The number of iron atoms per ferritin molecule is shown in Fig. 4.7 for the protein deficient and casein control animals for all three experiments performed with females.

In Experiment III the protein deficient animals stored 2,545 SD ± 156 iron atoms per molecule in comparison with the 2,326 SD ± 289 of the controls ($P > 0.05$, $t = 1.335$). In Experiment IV approximately similar values were observed for the protein deficient group (2,502 SD ± 131) and casein controls (2,489 SD ± 174) ($P > 0.05$, $t = 0.119$), whilst in the final trial a value of 2,479 SD ± 234 was observed for the protein deficient group compared with 2,262 SD ± 187 for the controls ($P > 0.05$, $t = 1.449$).

It is clear from these results that unlike their male counterparts, protein deficient females do not increase the number of iron atoms within the protein shell. It is

again seen that the number of iron atoms per molecule of ferritin for both protein deficient and protein replete animals is well below the theoretical maximum.

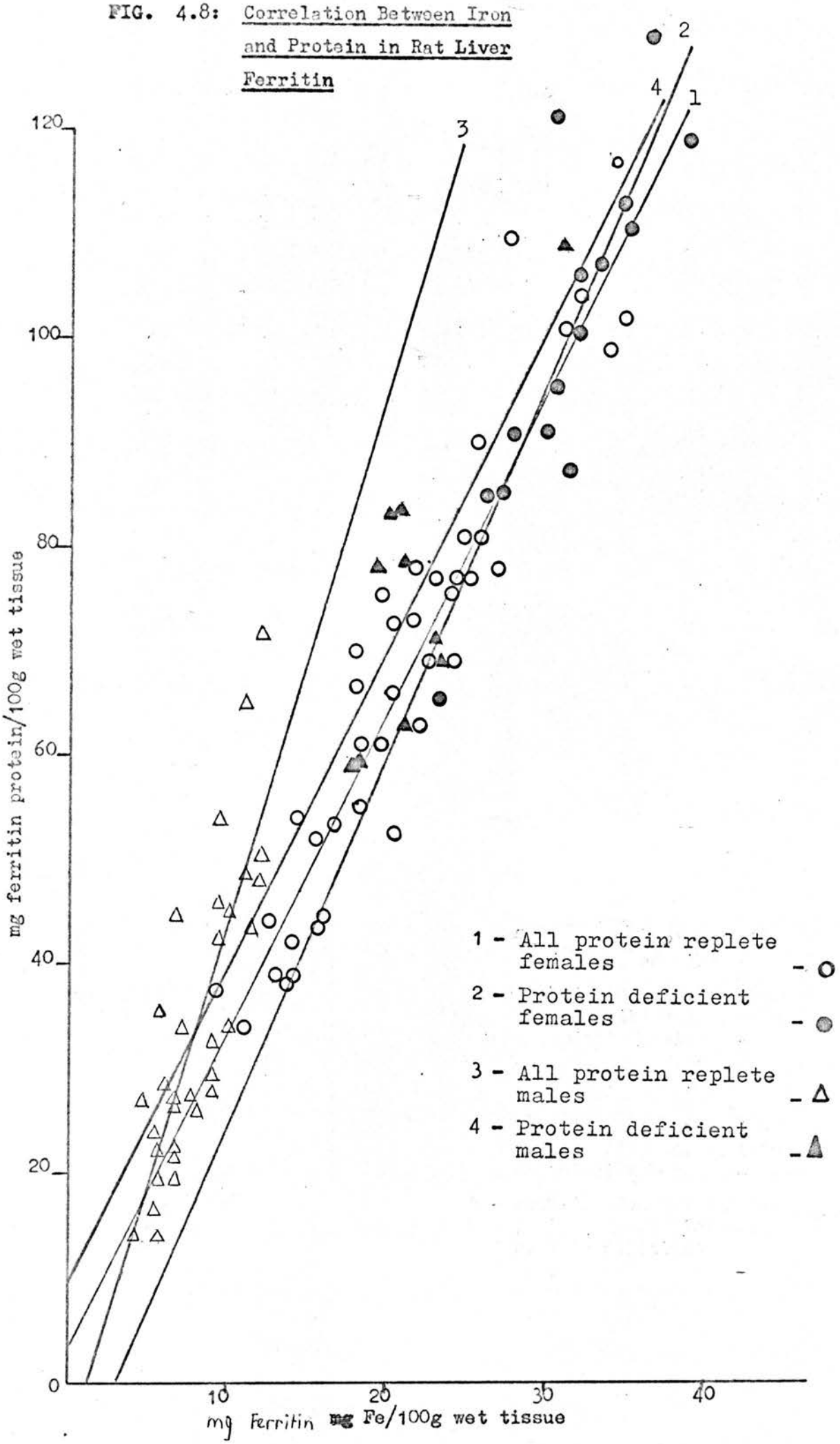
2. Statistical correlation between iron and protein

Close inspection of the individual results (Appendix II) showed that within any one group on a particular dietary regime there was a wide range in the values for ferritin iron and ferritin protein especially when expressed as a concentration of mg/100g wet tissue. It was therefore of interest to examine these results further to determine whether there was any closer relation between ferritin iron and ferritin protein than was disclosed by the simple statistical description already given.

Females:

The range in values for ferritin iron was 9.40-37.50 mg/100g wet tissue and that for ferritin protein was 33.91-116.77 mg/100g wet tissue for all protein replete females including those killed at the outset of the experiments. The average number of iron atoms per molecule protein for the individual animals ranged from about 2,000-3,000, with the greater number of values falling in a narrower range of 2,300-2,700. The individual protein contents plotted graphically against the ferritin iron content (Fig. 4.8) clearly indicated close proportionality and the regression line was calculated to be represented by the equation:

FIG. 4.8: Correlation Between Iron and Protein in Rat Liver Ferritin



$y = 3.06x (\pm 0.17) + 2.91 (\pm 3.70)$ ..... (1)

where the correlation coefficient $r = 0.947$, and y = ferritin protein (mg/100g wet tissue), x = ferritin iron (mg/100g wet tissue).

Such close correlation is surprising when it is recalled that the individual values were from animals of widely varying age maintained on qualitatively different dietary regimes, and in one instance (Experiment V) when the food intake was restricted.

For all the protein deficient females the ferritin iron content ranged from 23.40-39.00 mg/100g wet tissue and that of ferritin protein ranged from 65.44-129.64 mg/100g wet tissue. The average number of iron atoms per molecule protein ranged from about 2200-2800 with the greater number of values falling in the range of 2400-2600. When the individual protein contents were plotted against the corresponding iron content (Fig. 4.8) the regression line was calculated to be:

$y = 3.55x (\pm 0.58) - 11.18 (\pm 18.46)$ ..... (2)

where the correlation coefficient $r = 0.861$ and x and y are as previous.

This equation when compared with the one for protein adequate females (equation 1) suggests that with respect to the relationship between protein and iron, protein deficient females may belong to the same statistical population as normal animals.

Males:

The individual results for all protein replete males,

including those killed at the outset of the experiments (Appendix II) showed that ferritin iron content ranged from 4.10-12.00 mg/100g wet tissue (9.40-37.50 mg/100g wet tissue for females) and that for ferritin protein was 13.94-71.70 mg/100g wet tissue (33.91-116.77 mg/100g wet tissue for females). The average number of iron atoms per molecule protein showed a wider spread 1,100-2,700 than females (2,000-3,000) but a greater number of values fell within the narrower range of 1,300-2,000 which is distinctly lower than that of females (2,300-2,700). The ferritin protein content of the male livers has been plotted graphically against corresponding ferritin iron (Fig. 4.8) and a regression line has been calculated to be:

$$y = 5.00x\ (\pm\ 0.62) - 5.81\ (\pm\ 5.15)\ \ldots\ldots\ (3)$$

where the correlation coefficient $r = 0.821$ and y = ferritin protein (mg/100g wet tissue), x = ferritin iron (mg/100g wet tissue).

Comparison of this regression line with that for protein replete females (equation 1) clearly shows that the two regression lines are markedly different. This treatment of the results thus highlights a sex difference in the relationship of ferritin iron to ferritin protein in protein adequate animals.

The values of ferritin iron and ferritin protein (mg/100g wet tissue) for the protein deficient males ranged from 17.60-31.20 and 59.01-109.50 respectively,

and the average number of iron atoms per molecule protein ranged from about 1900-2600 (protein deficient females, 2200-2800). The ferritin protein content has been plotted graphically against the corresponding ferritin iron (Fig. 4.8) and a regression line was calculated to be:

$$y = 3.09x\ (\pm\ 0.85) + 8.78\ (\pm\ 18.52)\ \ldots\ldots\ (4)$$

where the correlation coefficient $r = 0.791$ and y = ferritin protein (mg/100g wet tissue), x = ferritin iron (mg/100g wet tissue).

This equation when compared with that for normal males (equation 3) clearly indicates that the protein deficient animals are markedly different from the controls. Furthermore equation (4) shows that in times of protein starvation iron storage in the male rat closely resembles that of females (equations 1 and 2).

The statistical treatment of the individual results which is summarised in Table 4.1 clearly reveals a certain regularity in the tendency of rats to store ferritin iron. For animals of considerably different ages and over a wide range of concentrations normal males use, within narrow limits, 5 mg protein for every 1 mg/100g wet tissue of extra iron deposited whereas normal females use only just over 3 mg protein for this purpose. Similarly both protein deficient females and males use approximately 3 mg protein to store every 1 mg/100g wet tissue of extra iron.

TABLE 4.1

Correlation Between Iron and Protein

in Rat Liver Ferritin

Animals	Regression Equation ($y = bx + a$) b(±SEM)	a(±SEM)	Correlation Coefficient (r)
○	3.06 (± 0.17)	2.91 (± 3.70)	0.947
●	3.55 (± 0.58)	-11.18 (± 18.46)	0.861
△	5.00 (± 0.62)	-5.81 (± 5.15)	0.821
▲	3.09 (± 0.85)	8.78 (± 18.52)	0.791

y = ferritin protein (mg/100g wet tissue), x = ferritin iron (mg/100g wet tissue), b = regression coefficient, a = regression constant

○ - all protein replete females ● - protein deficient females

△ - all protein replete males ▲ - protein deficient males

CHAPTER 5

DISCUSSION

Isolation of Ferritin

The high yields of ferritin obtained with the new procedure have been primarily achieved by deferring heat coagulation until after CM-cellulose chromatography. It was suggested (J.W. Drysdale, personal communication), that any ferritin which was denatured by heating the crude tissue homogenate at 70 C might renature on prolonged standing at 4 C and that losses of ferritin iron on lowering the pH and on chromatography might be avoided. This suggestion was not further investigated since the problem has been solved as already outlined (p.33). It is still not known with certainty, however, whether heating at this later stage is still without effect on the structure of the protein. Ishitani et al. (1975) have observed changes in circular dichroism reflecting protein unfolding when native apoferritin was heated in aqueous solution at 70 C. These changes were largely but not entirely reversed if heating was performed for less than 15 minutes followed by rapid cooling to room temperature.

The conditions employed for CM-cellulose chromatography in the new procedure have ensured that ferritin is eluted from the column as rapidly as possible as a single band and have thus minimised the chances of denaturation or of fractionation, whether real or artefactual, which might result from prolonged exposure to the ion exchange material. The yields from the column are high but they

are expressed as recovery of ferritin iron not ferritin protein. Since apoferritin and ferritin have similar electrostatic charges and because no previous treatments are performed which might entail a selective denaturation of apoferritin, it is reasonable to assume that the apoprotein migrates with and is recovered in the same fraction as that of the iron containing protein.

It has been shown (p.18) that on an analytical scale, ferritin and haemosiderin are quantitatively precipitated from a crude tissue homogenate by precipitation with 50 per cent saturated ammonium sulphate. In some instances during the isolation when ferritin was precipitated from a partially purified solution by the addition of an equal volume of saturated ammonium sulphate, the yields were less than 90 per cent (Table 2.7). Whilst this was partly attributed to a manipulatory error in handling large volumes it did suggest that apoferritin might be particularly at risk and be incompletely precipitated. Precipitation was, therefore, performed by adding solid ammonium sulphate to give a final saturation of 60 per cent. This greater degree of saturation and the smaller volumes handled facilitated a more complete recovery of ferritin iron and presumably also apoferritin, although it can not be categorically claimed that no apoferritin is lost. Nonetheless, it has been shown (p.42) that within the limits of reproducibility of the assay procedure, pure ferritin solutions are precipitated with 60

per cent saturated ammonium sulphate with no change in the iron to nitrogen ratio of the recovered precipitate; and this argues against loss of apoferritin, or low iron ferritin, under these conditions. It is thus considered that, in the absence of more direct evidence, precipitation by 60 per cent saturation with ammonium sulphate (added as solid) minimises the loss of apoferritin or low iron ferritin during the isolation.

An alternative isolation procedure, based on the principles of affinity chromatography, might well be employed to minimise the possibility of discrimination against different molecular populations. Marcus and Zinberg (1974) have reported the isolation of ferritin from human mammary and pancreatic carcinomas by means of antibody immunoadsorbents. The procedure used by these workers might be modified for the routine isolation of ferritin from a tissue homogenate. Basically, ferritin antibody would be covalently attached to Sepharose 4B and when a crude tissue homogenate (from which cellular debris is removed by a preliminary centrifugation) is passed through, only ferritin and apoferritin molecules are retained. These would then be recovered by passage of a suitable reagent, for example potassium thiocyanate at neutral pH (Marcus and Zinberg 1974) which dissociates the antigen-antibody complex. Unfortunately, it has not been possible to implement these ideas during this work.

Ferritin Iron and Protein in Protein Deficiency

Ferritin Iron:

In the case of male rats this study has confirmed the work of Hallgren (1953) and Achmed and Ramsay (1974) who observed that protein deficient males stored significantly more iron as ferritin than those fed a nutritionally adequate diet. However, in the present study when the younger males were used the absolute difference was not as marked as that observed by Hallgren (1953) and Achmed and Ramsay (1974). This might be partly attributable to the use of a different strain of rat, since it has been observed in this laboratory that there is considerable variation between different strains in the storage of non haem iron. For the particular strain used in this work, the younger animals fed the control diets showed a tendency to deposit extra ferritin iron during the five week experiment. Perhaps if the experiments had been extended to 7 weeks as in the study of Achmed and Ramsay (1974) the deposition of iron in the controls might have levelled off and a more marked difference between controls and protein deficient animals might have been observed. It might also be recalled that for technical and commercial reasons (p.46) the diets used in this work were not exactly identical to those used by Achmed and Ramsay (1974) and although unaware of any significant differences there might have been some. As noted previously (p.13),

Bjorklid and Helgeland (1970) observed that the male continued to build up iron stores up to at least 16 weeks of age.

Female rats are known to store iron at a greater rate and to a greater extent than males (Widdowson and McCance, 1948, Kaldor and Powell 1957, Bjorklid and Helgeland 1970, Linder et al. 1973). This study has shown that protein deficient females have deposited ferritin iron to approximately the same extent as the controls. There is thus a clear difference between the sexes in respect to their response to protein deficiency. Mature male rats normally maintain a constant level of stored iron, including ferritin (Achmed and Ramsay 1974), in the liver, but in protein deficiency the hepatic stores are substantially increased. Normal unbred females, on the other hand continue to increase their iron stores, but protein deficiency causes no further rise.

Ferritin protein:

Despite some fluctuation in the results, this study has shown that during an extended period of protein deprivation both male and female rats are able to accumulate ferritin protein in the liver to much the same levels as animals maintained on a nutritionally adequate diet. In Experiment II the group fed the control diet ad libitum stored significantly more than those pair fed the same diet but this latter group (4.68 mg SD $\pm$ 0.16) accumulated a similar amount as animals fed the laboratory stock diet

(4.86 mg SD ± 0.47). From this comparison and the results with females in Experiment V it appears that pair-feeding the diets had little influence on the overall result.

The protein deficient males have increased the number of iron atoms per ferritin molecule considerably above that of the control animals, whereas the protein deficient females have not shown this behaviour. Obviously a sex difference exists in that protein deficient males are economising in protein by accommmodating a greater number of iron atoms within the protein shell. Both protein deficient males and females stored approximately 2,500 iron atoms per protein molecule on average which is far removed from the maximum theoretical value of 4,300.

From this analysis it thus appears that protein adequate and protein deficient females and more remarkably, protein deficient males, all belong to the same statistical population. This suggests that in times of protein deficiency the hepatic iron storage metabolism of the male rat resembles that which is characteristic of the protein adequate female whereas that of the protein deficient female remains unaltered.

These conclusions have beendrawn from the study of only one particular strain of rat but there would appear to be no 'a priori' reason why they might not be generally valid. The sex difference which has been established in normal animals is in agreement with the observations of

Bjorklid and Helgeland (1970) who also reported that female liver ferritin contains more iron atoms per molecule protein than that of males of similar age. In contrast however, Linder et al. (1973) reported that although the female stores more liver ferritin iron than the male, the average number of iron atoms per molecule protein stored by the male was 1870 compared with a value of 1580 for the female but in a previous report (Linder and Munro 1972) an average value of 2990 iron atoms per molecule protein for the adult rat was calculated. No explanation was given for these divergent observations.

The statistical treatment of the individual results described in the preceding chapter has demonstrated a close relationship between ferritin iron and ferritin protein. In support of the previous conclusions it provides further evidence for a sex difference in normal animals: the increase in liver ferritin protein per unit increase in liver ferritin iron is greater for males than for females, or expressed in another form, the male uses more protein to store ferritin iron. Furthermore it illustrates even more clearly that in prolonged protein deficiency the altered ferritin metabolism of the male is such as to warrant its inclusion in the same statistical population as females.

Aspects of Liver Ferritin Metabolism in Protein Deficiency

The two major points emerging from this work are: i) the ability of both male and female rats to maintain apparently normal amounts of ferritin protein in the face of prolonged protein deficiency and ii) the increased efficiency with which iron is stored in ferritin in protein deficient males. With regard to the former, ferritin is strikingly different from many hepatic enzymes such as the mitochondrial oxidative enzymes succinic dehydrogenase and cytochrome C oxidase and lysosomal D-amino acid oxidase (Waino et al. 1953); xanthine oxidase and methyl butyrate esterase (Knox et al. 1956); histidase and urocanase (Enwonwu and Streebny 1970); urea cycle enzymes, ornithine transcarbamylase, argininosuccinate synthetase, argininosuccinase (Hoffenberg et al. 1971); all of which have been reported to have greatly decreased activities. Decreased levels of proteins secreted from the liver such as albumin (Wannemacher 1961, 1963, Theophilus and Barnes 1961, Kirsch et al. 1968, Morgan and Peters 1971, Hoffenberg et al. 1971) and transferrin (Morgan and Peters 1971, MacFarlane et al. 1970, Olsen 1975) have been observed in rats, dogs and humans.

It thus appears that ferritin might be unique among liver proteins during periods of prolonged protein starvation. This uniqueness may be partly attributed to its ability to sequester iron and prevent possible

cytotoxic effects that increased levels of this metal may exert during such times.

The present investigation has shown that although normal male and female rats store approximately similar proportions of liver non haem iron as ferritin (Fig. 4.2, 4.4), the males use more protein for this purpose. In protein malnutrition, however, the male responds quite differently from the female and tends to economize in the use of protein and assume the female characteristic. Such a response may in part be influenced by an altered metabolism of male sex hormones which are produced both in the testis and in the adrenal cortex (Harper 1973). In protein deficiency the pituitary gland secretes less adrenocorticotropic hormone (Munro 1964) which under normal conditions non specifically stimulates the synthesis of all adrenal steriods. Thus the testosterone output from this source could well be diminished during protein starvation. There is also strong evidence that testosterone secretion by the testis is also diminished. In the adult male rat the gonadotropin content of the pituitary is reduced by protein starvation (Srebnik et al. 1959, Leatham and Fischer 1959, Srebnik and Nelson 1962) as also are the circulating levels of follicle stimulating hormone and interstitial cell stimulating hormone (luteinising hormone)(Leatham and Fischer 1959). Platt et al.(1964) reported that the testes of male rats fed a non protein diet suffer great loss of interstitial (Leydig) cells which are responsible for

testosterone synthesis. From the evidence of decreased accessory organ weight, Leatham (1970) concluded that protein depletion reduced androgen production by adult male testis.

The mechanism by which lower levels of the androgens might alter ferritin metabolism in protein deficiency is not known. It might be speculated that a reduced level indirectly promotes increases in iron absorption and liver storage iron and that this stimulus serves to abolish the sex difference in protein deficiency. Linder and Munro (1973) have suggested that in normal conditions the sex difference in rat liver iron storage could be obliterated by parenteral administration of iron, and Widdowson and McCance (1948) reported that removal of the ovaries or testes also eliminated this difference. This postulated role of testosterone might be tested by administering the hormone to the male during the period of protein depletion and assessing whether the mode of iron storage is altered from that of a non treated protein deficient "control". Gonadotropins might also be administered since it has been reported that despite inanition the rat testis will respond to such treatment with a stimulation of the Leydig cells (Mulinos and Pomerantz 1941).

An average value of about 2,500 iron atoms per molecule has been consistently approached by female rats having widely different ferritin iron contents and by

both protein deficient males and females. The average value is well below the maximum theoretical one of 4,300 iron atoms per molecule and this suggests that the system, in addition to retaining its normal functions in protein deficiency, is subject to some regulatory control mechanism which is operating as efficiently in protein deficient animals as in normals. Figure 5.1 summarizes some of the essential features of ferritin metabolism and indicates some points worthy of further discussion.

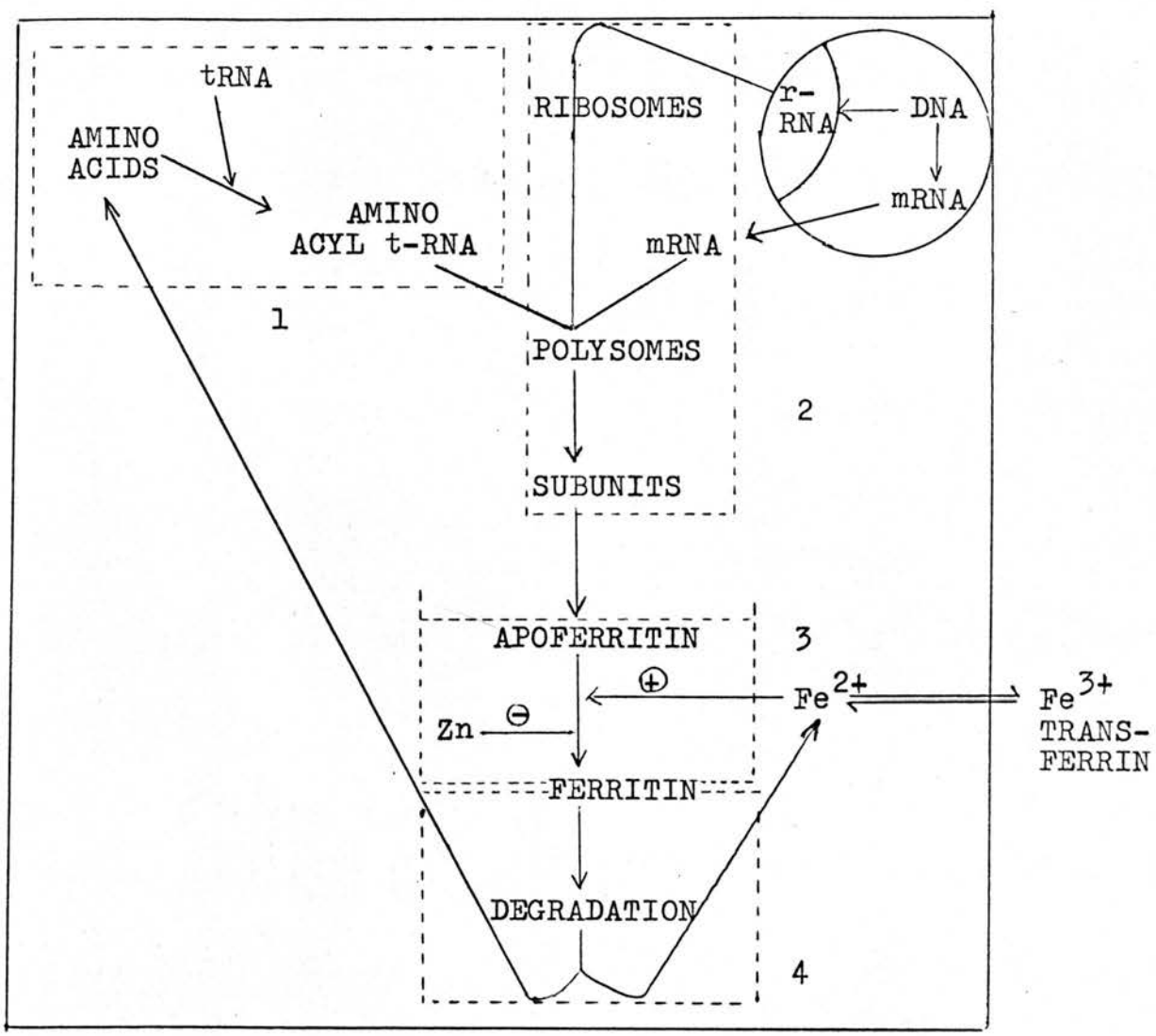
1. Protein deficiency and amino acid availability

Changes in the rates of either the synthesis of apoferritin or the degradation of ferritin might account for the accumulation of this protein in the livers of protein deficient animals since it is well established that the balance between the synthetic and degradative processes determines the amount of protein found at any one time in a cell or tissue. With reference to point '1' Fig. 5.1, the most obvious consequence of protein depletion is the elimination of a dietary source of amino acids. For continued synthesis a complete and ready supply of amino acids from alternative sources must be available and there is evidence to suggest that this might not be a serious drawback.

The catabolism of muscle protein may enable amino acid dependent functions of such organs as the liver to escape the most severe consequences of protein deficiency (Widdowson et al. 1960, Arroyave 1962, Viteri et al. 1964). Endocrine responses are associated with

FIG. 5.1

Scheme Summarizing Ferritin Metabolism in Hepatic Cell



LEGEND:

⊕ Indicates catalytic action of apoferritin $Fe^{2+} \longrightarrow Fe^{3+}$

⊖ Indicates inhibition of iron uptake by zinc.

Areas 1, 2, 3, 4 encompassed by, -----, represent aspects of ferritin metabolism discussed more fully in the text.

muscle catabolism at these times and act in such a way as to redistribute amino acids towards the liver (Munro 1964). Generally, insulin and anabolic hormones (growth hormone and the androgens) cause deposition of amino acids in muscle and lead to impairment of labile protein formation in the liver. Decreased concentrations of circulating insulin have been found within one week of feeding the rat a low protein diet (Young et al. 1973). In severe phases of protein calorie malnutrition in human subjects the level of circulating insulin is decreased (Hadden 1967). On the other hand, catabolic hormones (corticosteroids, thyroxine) cause preferential deposition of amino acids in the liver. Protein deficiency has been found to diminish secretion of growth hormone and gonadotrophic hormones from the anterior pituitary in the adult rat, but the thyroid stimulating hormone remains unaffected (Srebnik et al. 1959, Srebnik and Nelson 1962). Elevated blood cortisol levels have been found in children suffering from protein malnutrition (Alleyne and Young 1966, 1967). The increased cortisol and decreased insulin levels have led Gürson (1972) to suggest that such changes probably explain part of the alteration in protein metabolism during protein deprivation.

Evidence of an increased catabolism of muscle protein and a redistribution of amino acids thereof has been presented by Gan and Jeffay (1967) who showed that

protein catabolism in normal fed rats contributed approximately 50 per cent of the intracellular amino acid pool of the liver and 30 per cent of that of muscle. During the early stages of fasting as much as 90 per cent of the amino acids of the liver was derived from protein degradation. However, after depletion of the labile liver protein the muscle protein breakdown increased and supplied some 65 per cent of the intracellular amino acid pool of the muscle which in time maintained amino acid levels in other tissues including the liver. Waterlow and Stephen (1968) showed that in the rat the muscle eventually loses a greater percentage (58%) of initial protein content than the liver (50%) and considerably more in terms of total protein during 6 weeks of protein starvation. Adibi et al. (1973) measured both essential and non essential amino acid levels in skeletal muscle and liver after eight days of protein starvation. Generally the fall in hepatic concentration levelled off after the second day whereas decreases in muscle continued throughout the experimental period. Perhaps the most decisive evidence that an adequate pool of hepatic amino acids is maintained during prolonged protein starvation has been presented by Enwonwu and Streebny (1970). These workers measured free amino acids in the liver of adequately fed rats and those fed a protein deficient diet for 70 days; the amounts of the individual acids are shown in Table 5.1. It was also shown that the formation of urea was

TABLE 5.1

*Concentration of Free Amino Acids in the Liver of Adequately Fed and 70-day Protein Deficient Rats

Amino Acid	Diet	
	18% Lactalbumin	0.5% Lactalbumin
Alanine	2.59 ± 0.98	1.90 ± 0.99
Methionine	0.09 ± 0.02	0.05 ± 0.02
Lysine	0.88 ± 0.33	0.99 ± 0.72
Isoleucine	0.19 ± 0.01	0.07 ± 0.03
Leucine	0.34 ± 0.09	0.17 ± 0.09
Valine	0.32 ± 0.15	0.13 ± 0.05
Threonine	0.44 ± 0.06	0.84 ± 0.56
Phenylalanine	0.13 ± 0.02	0.07 ± 0.03
Histidine	0.56 ± 0.09	0.60 ± 0.25
Arginine	Trace	0.14 ± 0.03
Tyrosine	0.13 ± 0.04	0.09 ± 0.06
Aspartic	2.09 ± 1.20	1.41 ± 0.80
Glutamic	1.08 ± 0.24	1.44 ± 0.81
Serine	0.54 ± 0.11	4.39 ± 2.16
Asparagine/ Glutamine	8.02 ± 2.57	2.62 ± 1.72
Proline	0.19 ± 0.04	0.13 ± 0.06
Glycine	2.27 ± 0.07	2.07 ± 1.11

Concentration of amino acids expressed as μ mol/g liver.

*Table taken from Enwonwu C.O. and Streebny L.M. (1970)

greatly reduced - 13.97 (SD ± 3.17) μmol/g liver in normal animals, 3.72 (SD ± 1.21) μmol/g liver for those made protein deficient - and it has already been noted (p.72) that many hepatic enzymes including those of the urea cycle and those involved in amino acid catabolism are depressed in protein deficiency. Thus although the liver amino acid analyses indicate some fall, this may not be critical since the competition for these acids will be diminished because of reduced hepatic synthesis of secretory proteins. The available pool might then be perfectly adequate to support continued synthesis of the more essential intracellular proteins including apoferritin.

Given an adequate supply of amino acids, a prerequisite for the synthesis of apoferritin is a source of specific mRNA; this aspect is discussed in the following section (2). However, prior to translation and incorporation into the polypeptide chain, amino acids require to be activated and charged to specific t-RNA's. The effect of protein depletion on t-RNA appears to be little understood, but Gaetani et al. (1964), Stephen (1968), Horie and Ashida (1971) have all observed an increased activity in the liver amino acyl-tRNA ligases during prolonged protein deprivation. The effect of this change in enzyme activity on the availability of amino acyl-tRNA was not, however, established.

2. Protein deficiency and the capacity for Polypeptide Synthesis

In an earlier section (p. 7) the evidence that apoferritin synthesis is to some extent controlled by iron

was discussed. Reference to the graph , Fig .4.8 , suggests that in protein deficiency this role of iron is still a predominant factor and perhaps the decisive one. The precise nature of the stimulation of apoferritin synthesis by iron has not yet been fully established and the present work does not advance an understanding of the mechanism. Because its effect is not inhibited by actinomycin the iron is believed to act at the level of translation, presumably in the primary subunit synthesis. This could be achieved by suppressing some inhibitor of subunit synthesis or alternatively by stabilising nascent apoferritin subunits against rapid intracellular breakdown (Munro and Drysdale 1970). More recently Zahringer et al. (1975) have isolated polysomes and mRNA from the livers of control and iron-treated rats and have examined their relative ability to synthesize apoferritin in cell free systems. Synthesis was found to be 2 fold greater when polysomes or mRNA from iron treated rats were used and this led these workers to suggest that iron somehow increases the amount and/or the availability of apoferritin mRNA in polysomes even if it is not being newly synthesised. Whatever the mechanism by which iron exerts its effect it is obviously necessary that the individual components of the protein synthesising machinery remain fully functional for the continuation of apoferritin synthesis during protein deficiency. With regard to the

availability of mRNA during periods of protein deficiency, the picture is not clear. Kido et al.(1973) reported a decreased level of mRNA for serum albumin following 14 days of protein starvation. There is evidence for the existence of both a short-lived messenger and a long-lived one in liver and the former has been shown to be preferentially lost in protein deficiency (Wilson and Hoagland, 1967). It might well be that proteins of the liver having an essential role in maintaining the integrity of the cell are coded for by a long-lived messenger. Drysdale et al. (1968) have shown that after 4 days of protein deprivation the rat was able to respond to the administration of iron and synthesise apo-ferritin. These authors suggested that the mRNA was either stable or that a reduced amount was used more efficiently. The extra iron accumulated during prolonged protein deficiency might augment the efficiency of either one or both of these processes, but such effects might be relatively short term. Continuing synthesis of the mRNA during extended periods of protein deficiency should not be discounted since the increased rate of degradation of ribosomes in the cytoplasm might furnish the necessary pool of nucleotides and it has been shown that there is an apparent increase in RNA polymerase activity following 7 days of protein deprivation (Wannemacher et al.1971). Glucocorticoid-stimulated synthesis of mRNA has been positively demonstrated for two liver enzymes, tryptophan

1.3 dioxygenase, tyrosine aminotransferase (Kenney 1970), but perhaps the increased levels of cortisol observed during protein deficiency might have significant influence in regulating synthesis of mRNA specific for ferritin and other essential intracellular hepatic proteins. Such a hypothesis must, however, be regarded as highly speculative.

Another major factor in the cytoplasmic regulation of protein synthesis in general is the extent of polysome aggregation. The work of Fleck et al. (1965) showed that a complete mixture of amino acids was necessary for polysome aggregation and it was shown (Wunner et al. 1966) that omission of tryptophan caused a loss of the heavy polysome population and an accumulation of monosomes and oligosomes. Sox and Hoagland (1966), Staehelin et al. (1967) showed that polysomes increase in abundance soon after a meal of protein is fed to a fasting animal. Protein depletion has been shown to cause loss of RNA from the endoplasmic reticulum (Wikramanayake et al. 1953, Fawcett 1955, Bernhard and Rouiller 1956) and the nucleolus, the site of RNA synthesis, has been reported to be enlarged and to contain more RNA in rat liver (Stenram 1958). Gaetani et al. (1969) have observed a preferential breakdown of bound polysomes after 20 days of protein starvation. An increased ratio of free to membrane bound ribosomes in the liver cytoplasm of the protein depleted rat compared

with protein replete animals has been found (Strenram and Nordgren 1970, Enwonwu and Streebny 1970). Nordgren and Strenram (1972) have reported a shorter half-life for ribosomal RNA in protein-deficient rats.

Despite this adverse effect of protein depletion on the polysome population and especially the membrane bound polysomes, apoferritin synthesis may not be seriously impaired since it has been suggested that apoferritin is preferentially synthesised on unbound polysomes (Redman 1969, Hicks et al. 1969, Puro and Richter 1971). Linder et al. (1974) have recently proposed that membrane bound and free polysomes are involved in the synthesis of two different apoferritin subunits of molecular weight 19,000 and 13,500. These authors found that the smaller subunit was synthesised on unbound polysomes and that its synthesis was preferentially stimulated following administration of iron. Thus increased levels of iron in protein deficient rat liver and the reported loss of membrane bound polysomes (Gaetani et al. 1964) may significantly affect apoferritin synthesis in that preferential formation of the smaller subunit might be favoured. This latter course of action may in consequence economize on the free amino acid pool but at present any such idea should be considered highly tentative, since it implies that the subunit structure of apoferritin of protein deficient animals is substantially different from that of normals.

3. Protein deficiency and iron uptake by apoferritin

From the previous discussion it is possible that the cytoplasmic elements necessary for apoferritin synthesis may remain functional during protein deficiency and be influenced to some considerable extent by the presence of increased amounts of iron. It would be of interest to reflect on the relationship between zinc and iron metabolism in protein deficiency particularly since the work of Coleman and Matrone (1969) and Macara et al. (1973) has revealed that zinc inhibits the uptake of iron by apoferritin. Van Campen and House (1974) studied the effect on the retention of an oral dose of zinc and on the liver concentration of zinc and iron in male rats after feeding a low protein diet for 23 days. Routinely it was found that the low protein diet caused an increased concentration of iron in the liver, confirming the work of Hallgren (1953) and Achmed and Ramsay (1974), and a reduced concentration of zinc. It is, therefore, possible that a lower zinc concentration might make iron uptake more effective in protein deficiency. Whether this would result in a higher average iron content in ferritin would depend on the exact mechanism, but if it did it could only have applied to the males in this work. Unfortunately it has not been possible to make zinc measurements in any of the livers in this work.

4. Post translational stabilisation

It is possible that part of the iron effect may lie in post-translation stabilisation of either the subunit or the aggregated subunits. Stabilisation of either the

subunit or the aggregated subunits by conjugation with a prosthetic group could well serve to regulate the level of the protein in the cell and an example of this is provided by the enzyme tryptophan 1.3 dioxygenase. The accumulation of this enzyme in the liver following administration of tryptophan may be the result of a tryptophan-stimulated conjugation of apoenzyme with haematin to form the more stable holoenzyme (Knox and Piras 1967). Drysdale and Munro (1966) claimed to have demonstrated the stabilisation of newly synthesised apoferritin by incorporation of iron and this action of iron appears to be supported by the finding of Crichton (1971) that apoferritin is more susceptible than ferritin to proteolysis. Ove et al. (1972) have compared the half-lives of rat liver ferritins of differing iron contents. The protein with an average 1,030 iron atoms per molecule showed a half-life of 1.9 days compared with a half-life of 4.0 days for ferritin with an average 1,960 atoms per molecule. The same authors suggested that accumulation of liver ferritin with age appeared to be due to a decreased rate of degradation.

Stabilisation by iron would be expected to assume even greater significance during protein deficiency since there is evidence to suggest that the proteolytic activity of the liver is not decreased in such times. Ninjoor et al. (1969) have observed the presence of swollen or injured lysosomes in protein deficient (11 day) rat liver

and release of proteolytic cathepsins into the cytoplasm. Dallman and Manies (1973) studied protein turnover in the mitochondrial, microsomal, microsomal membrane, ribosomal and supernatant fractions of a rat liver homogenate of animals fed a low casein diet for 14 days and found no evidence to suggest a decrease in the average turnover of liver protein as a result of protein deficiency.

During the course of this work it has not been possible to measure the rates of synthesis or degradation which might have led to a fuller understanding of the mechanism of ferritin metabolism during prolonged protein depletion. The foregoing discussion has attempted to show that in theory the continued synthesis of apoferritin might well be possible in such times and that the role of iron might be decisive in this and in that of reducing the rate of degradation. These two processes acting together might then account for the maintenance of the apparently normal levels of ferritin protein which have been observed in protein deficient animals of both sexes.

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APPENDIX I

Preparation of Antiferritin Serum

The antiferritin serum was kindly donated by Dr. J.D. Cook (Department of Medicine, University of Washington, Seattle, Washington) who described its preparation as follows: 100 mg of six times re-crystallised horse ferritin in complete Freund adjuvant (0.5 ml) was injected, at weekly intervals for three weeks, into the rabbit in multiple subcutaneous sites. Two weeks later the animals was given a final sub-cutaneous injection of the same amount of ferritin diluted in saline. Two weeks later it was bled by cardiac puncture 50 ml daily times three. The anti-serum was deep frozen until use.

The potency of the antiserum was established in this laboratory. Although the limit was not determined, 1 ml of a 1-in-5 or a 1-in-10 dilution of the antiserum was found to satisfactorily precipitate up to 35μg of ferritin iron.

APPENDIX II

EXPERIMENT I

ANIMAL	BODY WEIGHT(g) Initial	BODY WEIGHT(g) Final	LIVER WEIGHT(g)	TOTAL NON HAEM IRON mg/100g Wet Tissue	TOTAL NON HAEM IRON mg/liver
■ Killed at outset	385	-	13.80	7.25	1.00
	405	-	15.20	8.85	1.35
	415	-	13.70	8.80	1.21
	420	-	13.30	11.50	1.53
	418	-	14.90	7.45	1.11
	mean	-	mean	mean	mean
	409±14	-	14.20±0.80	8.77±1.70	1.24±0.21
▲ Protein Deficient Diet	412	325	9.20	22.10	2.03
	406	300	8.70	25.50	2.22
	408	310	8.70	26.40	2.30
	380	280	8.10	20.80	1.68
	376	280	9.30	26.50	2.46
	mean	mean	mean	mean	mean
	396±17	299±19	8.80±0.50	24.26±2.63	2.14±0.30
△ Casein Diet	404	402	13.50	8.50	1.15
	420	422	14.90	12.80	1.91
	428	435	14.00	13.60	1.90
	438	450	14.20	9.50	1.35
	430	410	11.60	14.80	1.72
	mean	mean	mean	mean	mean
	422±13	424±19	13.60±1.20	11.84±2.71	1.61±0.34
X Regular Laboratory Diet	442	478	15.20	9.75	1.48
	414	440	16.90	8.15	1.38
	404	405	15.00	8.05	1.21
	412	445	13.00	8.80	1.14
	423	435	12.20	11.05	1.35
	mean	mean	mean	mean	mean
	419±15	441±26	14.50±1.90	9.16±1.26	1.31±0.14

EXPERIMENT I (Cont.)

	FERRITIN IRON mg/100g Wet Tissue	FERRITIN IRON mg/Liver	FERRITIN PROTEIN mg/100g Wet Tissue	FERRITIN PROTEIN mg/Liver	Fe:N RATIO (g/g)	Fe ATOMS/ MOLE PROTEIN
■	5.65	0.78	19.42	2.68	1.82:1	2288
	7.60	1.15	27.50	4.18	1.72:1	2162
	6.75	0.92	26.60	3.64	1.58:1	1986
	9.20	1.22	27.67	3.68	2.07:1	2602
	5.35	0.80	23.83	3.55	1.41:1	1772
	mean	mean	mean	mean		mean
	6.91±1.56	0.97±0.20	25.00±3.48	3.55±0.54		2162±313
▲	17.60	1.62	59.13	5.44	1.86:1	2338
	23.00	2.00	71.15	6.19	2.02:1	2539
	21.00	1.83	63.22	5.50	2.08:1	2615
	18.00	1.46	59.09	4.78	1.91:1	2401
	23.30	2.17	69.14	6.43	2.11:1	2652
	mean	mean	mean	mean		mean
	20.58±2.68	1.81±0.29	64.35±5.60	5.67±0.66		2509±136
Δ	6.50	0.88	27.19	3.67	1.50:1	1886
	9.00	1.34	29.40	4.38	1.91:1	2401
	10.10	1.41	34.29	4.80	1.84:1	2313
	7.20	1.02	33.80	4.80	1.33:1	1672
	11.40	1.32	43.36	5.03	1.64:1	2061
	mean	mean	mean	mean		mean
	8.84±2.02	1.19±0.23	33.61±6.21	4.54±0.54		2067±300
X	6.70	1.02	19.34	2.94	2.17:1	2728
	5.50	0.93	22.30	3.77	1.54:1	1936
	5.30	0.80	16.60	2.49	2.01:1	2527
	6.50	0.85	21.62	2.81	1.89:1	2376
	7.90	0.96	25.98	3.17	1.89:1	2376
	mean	mean	mean	mean		mean
	6.38±1.04	0.91±0.09	21.17±3.49	3.04±0.48		2389±291

EXPERIMENT II

ANIMAL	BODY WEIGHT(g) Initial	BODY WEIGHT(g) Final	LIVER WEIGHT(g)	TOTAL NON HAEM IRON mg/100g Wet Tissue	TOTAL NON HAEM IRON mg/liver
■ Killed at outset	295	-	10.20	9.30	0.95
	285	-	11.70	5.50	0.64
	290	-	10.40	6.30	0.66
	290	-	9.00	8.60	0.77
	280	-	9.00	8.60	0.77
	mean	-	mean	mean	mean
	288±6	-	10.10±1.10	7.66±1.66	0.76±0.12
▲ Protein Deficient Diet	298	251	7.20	24.00	1.73
	286	232	6.30	23.30	1.47
	285	223	6.00	33.70	2.02
	297	252	6.80	24.60	1.67
	296	250	6.60	23.20	1.53
	mean	mean	mean	mean	mean
	292±6	242±13	6.60±0.50	25.76±4.47	1.68±0.22
◬ Casein Diet (pair fed)	298	326	9.90	12.50	1.24
	285	288	9.40	15.30	1.44
	285	301	10.00	13.80	1.38
	295	295	8.60	17.50	1.51
	295	284	10.00	14.30	1.43
	mean	mean	mean	mean	mean
	292±6	299±17	9.60±0.60	14.68±1.87	1.40±0.10
△ Casein Diet (ad lib)	295	324	10.50	11.50	1.20
	297	375	9.40	13.30	1.25
	290	349	11.80	12.60	1.48
	290	337	10.60	15.30	1.62
	285	317	11.60	12.30	1.42
	mean	mean	mean	mean	mean
	291±5	340±23	10.80±1.00	13.00±1.44	1.39±0.17
X Regular Laboratory Diet	295	382	15.40	7.90	1.22
	320	384	14.00	6.90	0.97
	350	430	12.90	8.30	1.07
	315	387	15.10	5.90	0.89
	330	422	15.00	11.20	1.68
	mean	mean	mean	mean	mean
	322±20	401±23	14.50±1.00	8.04±2.00	1.17±0.31

EXPERIMENT II (Cont.)

	FERRITIN IRON mg/100g Wet Tissue	FERRITIN IRON mg/Liver	FERRITIN PROTEIN mg/100g Wet Tissue	FERRITIN PROTEIN mg/Liver	Fe:N RATIO (g/g)	Fe ATOMS/ MOLE PROTEIN
■	6.60	0.67	22.35	2.28	1.84:1	2313
	4.40	0.51	14.53	1.70	1.88:1	2363
	4.10	0.43	13.94	1.45	1.85:1	2326
	6.20	0.56	26.11	2.35	1.49:1	1873
	6.60	0.59	-	-	-	-
	mean	mean	mean	mean		mean
	5.58±1.23	0.55±0.09	19.23±5.98	1.95±0.44		2219±231
▲	21.00	1.51	78.47	5.65	1.67:1	2099
	20.30	1.28	83.49	5.26	1.52:1	1911
	31.20	1.87	109.50	6.57	1.78:1	2238
	20.80	1.41	83.68	5.69	1.55:1	1948
	19.20	1.27	78.03	5.15	1.54:1	1936
	mean	mean	mean	mean		mean
	22.50±4.93	1.47±0.25	86.63±13.06	5.66±0.33		2026±139
◬	9.30	0.92	46.06	4.56	1.26:1	1584
	11.80	1.11	50.53	4.75	1.46:1	1835
	10.20	1.02	45.20	4.52	1.41:1	1772
	13.60	1.17	-	-	-	-
	10.90	1.09	48.70	4.87	1.40:1	1760
	mean	mean	mean	mean		mean
	11.16±1.64	1.06±0.10	47.62±2.45	4.68±0.16		1738±108
△	9.50	1.00	54.09	5.68	1.10:1	1383
	11.80	1.11	48.08	4.52	1.28:1	1609
	11.20	1.32	65.25	7.70	1.07:1	1345
	12.00	1.27	71.70	7.60	1.04:1	1308
	9.70	1.13	42.59	4.94	1.43:1	1798
	mean	mean	mean	mean		mean
	10.84±1.17	1.17±0.13	56.34±12.01	6.27±1.29		1489±209
X	6.20	0.95	28.57	4.40	1.34:1	1684
	5.70	0.80	36.07	5.05	0.99:1	1244
	6.50	0.84	44.73	5.77	0.91:1	1144
	4.60	0.69	26.95	4.07	1.06:1	1332
	8.90	1.34	33.27	4.99	1.68:1	2112
	mean	mean	mean	mean		mean
	6.38±1.58	0.92±0.25	33.92±7.06	4.86±0.47		1503±358

EXPERIMENT III

ANIMAL	BODY WEIGHT(g) Initial	BODY WEIGHT(g) Final	LIVER WEIGHT(g)	TOTAL NON HAEM IRON mg/100g Wet Tissue	TOTAL NON HAEM IRON mg/liver
□ Killed at outset	189	-	6.80	18.10	1.23
	192	-	6.30	17.90	1.13
	200	-	6.40	13.90	0.89
	189	-	7.00	15.50	1.09
	194	-	6.10	11.90	0.73
	mean	-	mean	mean	mean
	193±5	-	6.50±0.30	15.46±2.65	1.01±0.20
● Protein Deficient Diet	200	167	5.70	27.00	1.54
	204	164	4.70	34.40	1.62
	206	168	5.30	36.50	1.93
	202	150	4.70	33.70	1.58
	200	161	5.80	31.40	1.82
	mean	mean	mean	mean	mean
	202±3	162±7	5.20±0.50	32.60±3.62	1.70±0.16
○ Casein Diet	194	203	6.50	42.50	2.76
	194	228	6.50	33.10	2.15
	190	199	6.10	23.50	1.43
	192	210	6.50	32.50	2.18
	205	225	7.10	25.20	1.79
	mean	mean	mean	mean	mean
	195±6	213±13	6.50±0.40	31.36±7.55	2.06±0.35
X Regular Laboratory Diet	203	260	9.20	22.60	2.08
	194	215	7.60	39.00	2.96
	208	250	7.30	25.00	1.83
	198	230	7.90	27.00	2.14
	200	234	7.80	27.40	2.13
	mean	mean	mean	mean	mean
	201±5	238±18	8.00±0.60	28.20±6.33	2.22±0.43

EXPERIMENT III (Cont.)

	FERRITIN IRON mg/100g Wet Tissue	FERRITIN IRON mg/Liver	FERRITIN PROTEIN mg/100g Wet Tissue	FERRITIN PROTEIN mg/Liver	Fe:N RATIO (g/g)	Fe ATOMS/ MOLE PROTEIN
	13.70	0.93	38.09	2.59	2.25:1	2828
	14.00	0.88	39.05	2.46	2.24:1	2817
	11.00	0.70	33.91	2.17	2.03:1	2552
□	13.20	0.92	38.86	2.72	2.13:1	2677
	9.40	0.57	37.38	2.28	1.57:1	1993
	mean	mean	mean	mean		mean
	12.26±1.98	0.80±0.20	37.46±2.09	2.44±0.23		2513±344
	23.40	1.33	65.44	3.73	2.24:1	2817
	27.40	1.29	85.32	4.01	2.01:1	2527
●	31.80	1.69	100.57	5.33	1.98:1	2489
	28.30	1.33	91.28	4.29	1.94:1	2439
	26.50	1.54	85.17	4.94	1.95:1	2451
	mean	mean	mean	mean		mean
	27.48±3.04	1.44±0.23	85.56±12.87	4.46±0.68		2545±156
	37.50	2.44	116.77	7.59	2.01:1	2527
	27.00	1.76	78.31	5.09	2.16:1	2715
○	20.50	1.25	72.79	4.44	1.76:1	2212
	27.00	1.81	109.54	7.12	1.59:1	1999
	21.50	1.53	77.89	5.53	1.73:1	2175
	mean	mean	mean	mean		mean
	26.70±6.75	1.76±0.44	91.06±20.45	5.95±1.35		2326±289
	18.40	1.69	55.00	5.06	2.09:1	2627
	32.50	2.47	-	-	-	-
X	22.10	1.61	99.32	7.25	2.13:1	2677
	21.60	1.71	-	-	-	-
	23.00	1.79	63.21	4.93	2.17:1	2728
	mean	mean	mean	mean		mean
	23.52±5.31	1.85±0.35	72.51±19.25	5.75±1.30		2677±51

EXPERIMENT IV

ANIMAL	BODY WEIGHT(g) Initial	BODY WEIGHT(g) Final	LIVER WEIGHT(g)	TOTAL NON HAEM IRON mg/100g Wet Tissue	TOTAL NON HAEM IRON mg/liver
□ Killed at outset	221	-	6.70	19.20	1.29
	218	-	6.50	18.30	1.19
	212	-	7.50	16.40	1.23
	216	-	7.00	16.80	1.18
	246	-	6.90	20.80	1.44
	mean 222±25	-	mean 6.90±0.40	mean 18.30±1.80	mean 1.27±0.11
● Protein Deficient Diet	222	152	4.90	45.00	2.21
	270	206	7.00	35.50	2.49
	225	167	5.10	39.40	2.01
	230	176	6.30	39.70	2.50
	235	173	5.50	35.80	1.97
	mean 236±19	mean 175±20	mean 5.80±0.90	mean 39.08±3.84	mean 2.24±0.26
○ Casein Diet	216	223	6.60	48.50	3.20
	213	213	6.10	35.30	2.15
	220	222	6.70	26.20	1.76
	225	235	6.90	23.00	1.59
	228	237	7.40	28.00	2.07
	mean 220±6	mean 226±10	mean 6.70±0.50	mean 32.20±10.17	mean 2.15±0.63
X Regular Laboratory Diet	225	238	7.70	30.20	2.33
	275	285	11.40	28.40	3.24
	220	229	9.20	26.10	2.40
	214	220	7.70	25.80	1.99
	220	224	7.90	22.50	1.78
	mean 231±25	mean 239±26	mean 8.80±1.60	mean 26.60±2.91	mean 2.35±0.56

EXPERIMENT IV (Cont.)

	FERRITIN IRON mg/100g Wet Tissue	FERRITIN IRON mg/Liver	FERRITIN PROTEIN mg/100g Wet Tissue	FERRITIN PROTEIN mg/Liver	Fe:N RATIO (g/g)	Fe ATOMS/ MOLE PROTEIN
	15.60	1.05	43.88	2.94	2.23:1	2803
	16.00	1.04	44.62	2.90	2.24:1	2816
□	14.00	1.05	42.13	3.16	2.08:1	2615
	15.50	1.09	52.00	3.64	1.87:1	2351
	18.30	1.26	61.30	4.23	1.86:1	2338
	mean	mean	mean	mean		mean
	15.88±1.55	1.10±0.09	48.79±7.95	3.37±0.56		2585±233
	39.00	1.91	118.78	5.82	2.05:1	2577
	30.20	2.11	91.57	6.41	2.11:1	2652
●	33.40	1.70	107.25	5.47	1.94:1	2439
	35.50	2.24	110.79	6.98	2.01:1	2527
	30.80	1.69	121.64	6.69	1.84:1	2313
	mean	mean	mean	mean		mean
	33.78±3.60	1.93±0.25	110.00±11.84	6.27±0.62		2502±131
	35.00	2.31	102.27	6.75	2.14:1	2690
	25.60	1.56	90.00	5.49	1.78:1	2237
○	20.50	1.37	66.27	4.44	1.93:1	2426
	18.80	1.30	59.42	4.10	1.98:1	2489
	25.50	1.89	77.16	5.71	2.07:1	2602
	mean	mean	mean	mean		mean
	25.08±5.89	1.69±0.42	79.02±17.40	5.30±1.06		2489±174
	25.20	1.94	80.78	6.22	1.95:1	2451
	26.40	3.00	81.05	9.24	2.03:1	2552
X	24.50	2.25	75.32	6.93	2.03:1	2552
	23.90	1.84	68.83	5.30	2.17:1	2728
	20.50	1.62	52.78	4.17	2.43:1	3055
	mean	mean	mean	mean		mean
	24.10±2.22	2.13±0.54	71.75±11.72	6.37±1.91		2668±238

EXPERIMENT V

ANIMAL	BODY WEIGHT(g) Initial	BODY WEIGHT(g) Final	LIVER WEIGHT(g)	TOTAL NON HAEM IRON mg/100g Wet Tissue	TOTAL NON HAEM IRON mg/liver
□ Killed at outset	224	-	7.80	16.70	1.30
	235	-	8.70	23.60	2.05
	236	-	8.80	24.80	2.18
	238	-	8.50	15.70	1.33
	231	-	7.00	20.80	1.46
	mean 233±6	-	mean 8.20±0.86	mean 20.32±4.05	mean 1.66±0.42
● Protein Deficient Diet	225	176	5.30	42.00	2.23
	200	165	5.50	46.60	2.56
	210	172	5.00	37.60	1.88
	208	158	5.10	44.60	2.28
	210	166	5.00	38.80	1.94
	mean 211±9	mean 167±7	mean 5.20±0.20	mean 41.92±3.80	mean 2.18±0.27
○ Casein Diet	195	214	9.40	24.60	2.31
	215	215	8.20	23.30	1.91
	215	222	7.90	44.00	3.48
	200	208	7.80	41.60	3.24
	215	247	8.70	29.50	2.57
	mean 208±10	mean 221±15	mean 8.40±0.70	mean 32.60±9.60	mean 2.70±0.65
X Regular Laboratory Diet	217	233	8.00	20.80	1.66
	218	224	8.10	31.80	2.58
	233	238	9.20	29.50	2.71
	225	225	8.10	22.00	1.78
	228	260	8.90	30.30	2.70
	mean 224±7	mean 236±15	mean 8.50±0.60	mean 26.88±5.08	mean 2.29±0.52

EXPERIMENT V (Cont.)

	FERRITIN IRON mg/100g Wet Tissue	FERRITIN IRON mg/Liver	FERRITIN PROTEIN mg/100g Wet Tissue	FERRITIN PROTEIN mg/Liver	Fe:N RATIO (g/g)	Fe ATOMS/ MOLE PROTEIN
	14.50	1.13	54.23	4.23	1.67:1	2099
	19.80	1.72	60.92	5.30	2.03:1	2552
□	19.80	1.74	-	-	-	-
	13.80	1.17	44.11	3.75	1.95:1	2451
	17.00	1.19	-	-	-	-
	mean	mean	mean	mean		mean
	16.98±2.84	1.39±0.31	53.08±8.46	4.43±0.64		2367±238
	32.20	1.71	106.66	5.65	1.89:1	2376
	36.80	2.02	129.64	7.13	1.77:1	2225
●	31.70	1.59	87.60	4.38	2.27:1	2853
	35.00	1.79	113.73	5.80	1.93:1	2426
	30.50	1.53	95.60	4.78	2.00:1	2514
	mean	mean	mean	mean		mean
	33.24±2.58	1.73±0.19	106.65±16.31	5.55±1.06		2479±234
	18.00	1.69	66.49	6.25	1.69:1	2124
	19.60	1.61	75.73	6.21	1.60:1	2011
○	32.10	2.54	104.05	8.22	1.93:1	2426
	31.40	2.45	101.67	7.93	1.93:1	2426
	23.00	2.00	77.70	6.76	1.85:1	2325
	mean	mean	mean	mean		mean
	24.82±6.58	2.06±0.42	85.13±16.75	7.07±0.94		2262±187
	16.60	1.33	53.25	4.26	1.95:1	2451
	24.50	1.98	77.16	6.25	1.98:1	2489
X	22.30	2.01	73.04	6.72	1.87:1	2351
	18.00	1.46	70.00	5.67	1.61:1	2024
	22.60	2.01	68.87	6.13	2.05:1	2577
	mean	mean	mean	mean		mean
	20.80±3.34	1.76±0.33	68.46±9.09	5.81±0.94		2378±214

APPENDIX III

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Storage of Iron as Ferritin in the Liver of the Protein-Deficient Rat

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Prolonged protein deficiency in the male rat causes increased deposition of iron in the liver and spleen (Hallgren, 1953), and the normal proportion of the additional iron is stored as ferritin (Achmed & Ramsay, 1974) even after 8 weeks of protein deprivation. Natural ferritin contains molecules with a wide spread of iron content, and it was decided to determine whether the protein-deficient rat maintains more ferritin protein or produces molecules with a higher average iron content. There seems no reason *a priori* why both mechanisms should not operate.

Ferritin was isolated from the livers of experimental rats by a procedure modified from that of Drysdale & Munro (1965). High yields (>70%) are regularly obtained, so that the chance of discriminating against ferritin of either high or low iron content is diminished. The iron and nitrogen contents of the electrophoretically pure product were used with separately determined values for the ferritin iron content of the liver to calculate the weight of ferritin protein in the whole liver and the average number of iron atoms per molecule. Both male and female rats were used because female rats normally store iron at a greater rate than males (Widdowson & McCance, 1948), but their response to protein deficiency has not been studied.

Mature animals in narrow weight ranges were fed for 5 weeks on diets similar to those used by Bethard *et al.* (1958). The control diet provided 22% protein as casein, which was omitted from the deficient diet. The daily supply of iron in the diets was 3–4mg/rat. Since the animals ate less of the protein-deficient diet than the normal one, pair-fed controls were included in some experiments. The animals were allowed free access to water and were weighed at weekly intervals. Males fed *ad lib* on the adequate diet grew nearly as much as animals on the laboratory stock diet. Weight loss was rapid on the deficient diet, whereas pair-fed controls only maintained their weight. The behaviour of the females was similar, except that on the normal diet they gained much less weight than males.

Ferritin iron was determined in a dilute (1+9) liver homogenate by precipitation of haemosiderin at pH4.8, precipitation of ferritin by addition of $(NH_4)_2SO_4$ to give 60% saturation and measurement of iron in the precipitate by the dipyridyl method. The results agreed well with those obtained by the analytical CM-cellulose method (Drysdale & Ramsay, 1965). Ferritin was isolated from the homogenate after pH4.8 precipitation by CM-cellulose chromatography, heat coagulation of the eluate at 70°C, precipitation by 60% saturation with $(NH_4)_2SO_4$ and gel filtration on Sephadex G-200. The heat-coagulation step was found to be necessary, but the overall yield was substantially increased by deferring it until after the ion-exchange step. Nitrogen was determined volumetrically after micro Kjeldahl digestion and separation of the ammonia by the Conway micro diffusion technique.

Table 1 summarizes the results of five experiments. More ferritin iron was found in the livers of protein-deficient male rats than in control animals (two experiments, $P<0.01$, $0.02<P<0.05$), but no significant difference was seen in three experiments on females. Liver non-haem and ferritin iron had, however, increased by 80–100% in protein-deficient as well as in control females on the experimental diets. Protein-deficient males gave ferritin containing 20–30% more iron atoms per molecule (two experiments,